

**Factors enhancing adherence of toxigenic  
bacteria to epithelial cells in relation to  
Sudden infant death syndrome**

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## **Declaration**

I declare here that the work for this thesis was carried out by myself or under my direct supervision.

Edinburgh, October 1994

Abdulrahman T Saadi

I dedicate this work to my father Towfeeq, my mother Halima, my wife Awaz  
and the memory of my uncle Najeeb

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## Abbreviations

|                       |  |
|-----------------------|--|
| <b>BEC</b>            | Buccal epithelial cells                                    |
| <b>BI</b>             | Binding index  |
| <b>BSA</b>            | Bovine serum albumin                                       |
| <b>CSOM</b>           | Confocal scanning optical microscopy                       |
| <b>EDTA</b>           | Ethylenediamine tetra acetic acid                          |
| <b>ELISA</b>          | Enzyme linked immunosorbent assay                          |
| <b>EPICS</b>          | Electronically programmable individual cell sorter         |
| <b>FITC</b>           | Fluorescein isothiocyanate                                 |
| <b>Gal</b>            | Galactose  |
| <b>GlcNac</b>         | N-acetylglucosamine  |
| <b>GalNac</b>         | N-acetylgalactosamine                                      |
| <b>IFN</b>            | Interferon   |
| <b>IL</b>             | Interleukin  |
| <b>Le<sup>a</sup></b> | Lewis <sup>a</sup>   |
| <b>Le<sup>b</sup></b> | Lewis <sup>b</sup>   |
| <b>Le<sup>x</sup></b> | Lewis <sup>x</sup>   |
| <b>LPS</b>            | Lipopolysaccharides  |
| <b>NO</b>             | Nitric oxide   |
| <b>OD</b>             | Optical density  |
| <b>PBS</b>            | Phosphate-buffered saline                                  |
| <b>RSV</b>            | Respiratory syncytial virus                                |
| <b>SDS-PAGE</b>       | Sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| <b>SIDS</b>           | Sudden infant death syndrome                               |
| <b>TNF</b>            | Tumour necrosis factor                                     |
| <b>TSS</b>            | Toxin shock syndrome                                       |
| <b>TSST-1</b>         | Toxin shock syndrome toxin-1                               |

# Abstract

Sudden infant death syndrome (SIDS) is the most common cause of postperinatal mortality in the developed world. The most consistent epidemiological finding is that the peak incidence of these deaths occurs between 2-4 months. This study tests the hypothesis that the Lewis<sup>a</sup> antigen expressed by 80-90 % of infants in the 2-4 month age range is one of the host receptors for two toxigenic bacteria species suggested to be involved in cot death, *Staphylococcus aureus* and *Bordetella pertussis*. Although respiratory viruses are often isolated from SIDS infants, there is no direct evidence for their involvement in these deaths; however, this study examined the effect of virus infection on binding of *S. aureus* and *B. pertussis* to epithelial cells in culture.

By flow cytometry, binding of three toxigenic strains of *S. aureus* to cells from non-secretors was significantly greater than to cells of secretors. Pretreatment of epithelial cells with monoclonal anti-Lewis<sup>a</sup>, anti-type 1 precursor or anti-Lewis<sup>x</sup> significantly reduced bacterial binding; but binding of *S. aureus* was significantly correlated only with the amount of Lewis<sup>a</sup> present on the epithelial cells. Binding of *B. pertussis* to epithelial cells was also significantly inhibited by pre-treatment of the cells with anti-Lewis<sup>a</sup> or anti-Lewis<sup>x</sup>. A 67 kDa protein was isolated from cell membrane preparations of *S. aureus* (NCTC 10655) by affinity adsorption with synthetic Lewis<sup>a</sup> antigen conjugated to Synsorb beads. Pre-treatment of BEC with the purified protein reduced binding of staphylococcal strains to a greater extent than with the material not bound to the Synsorb beads.

Respiratory Syncytial Virus (RSV) infects about 50% of infants by the first year of life and it is often isolated from infants with SIDS. RSV-infected HEp-2 cells bound significantly more *S. aureus* or *B. pertussis* than uninfected cells.

The findings of this study help to explain some of the epidemiological factors associated with SIDS, the age range affected and the winter peak of these deaths. The significance of these findings is discussed in the context of recent recommendations for infant care procedures, changes in infant immunization schedules and the steady decline in SIDS observed since 1990.

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# **Chapter 1**

## **General introduction**

In spite of the improvement in living conditions and general health of the population world-wide, a significant proportion of infants die suddenly without apparent cause of death. Many epidemiological and pathological studies indicate infectious agents might be involved in the aetiology of these infant deaths.

The aim of this chapter is to review the current concepts of the epidemiology and aetiology of sudden infant death syndrome (SIDS), in particular the toxigenic bacteria which have been implicated in SIDS, in the context of genetic, developmental and environmental factors associated with susceptibility to infectious agents.

### **1.1 SIDS**

The term "cot death" was first used by Barret [1954] to describe the unexpected death of a healthy baby during sleep. Some used the synonym "crib death". Cot death includes sudden unexpected infant death. The causes of some might be explained, some partially explained and others unexplained. Those for which the causes are totally unexplained or partially explained are classified as sudden infant death syndrome.

The term SIDS is a pathological diagnosis of exclusion. The most accepted definition was proposed by Beckwith [1970]: "The sudden death of an infant or young child which is unexpected by history, and in whom a thorough autopsy examination fails to demonstrate an adequate cause of death". The recent revised definition for SIDS is: "The sudden death of an infant under one year of age which remains unexplained after a thorough case investigation, including performance of a complete autopsy,

examination of the death scene, and review of the clinical history [Willinger *et al.*, 1991]". Bartholomew *et al.* [1987] found a definite cause of death in about 8% of cot deaths and possible contributory factors in a further 22%. They emphasised that there is no clear epidemiological distinction between true SIDS and those deaths which were explainable.

## **1.2 Epidemiology of SIDS**

SIDS is the major cause of death in infancy between the first week of life and one year. It has a characteristic age distribution with a clear peak around the third and the fourth months of life, and about 80% of the cases occur during the first six months of age [Frogatt *et al.*, 1971].

Despite a steady decline in infant mortality in the UK over the last decades from 14.4 per 1000 live births in 1975 to 8.3 in 1989, the incidence of SIDS remained at about 2 per 1000 live births [Finlay and Rudd, 1993]. There was a drop in the annual death rate of SIDS during the last few years. These figures are similar to those reported for Australia and the United States. The highest incidence of SIDS has been reported from New Zealand and the lowest from Hong Kong (Table 1.1). There are more males among the sudden unexplained deaths than among others in the same age range who died of other causes [Jorgensen *et al.*, 1979; Gibson, 1992].

### **1.2.1 Weather and season in relation to SIDS**

There is a winter peak of SIDS cases in the UK [Carpenter and Gardner, 1990] and South Australia [Beal, 1983], but this pattern is not found in Scandinavia [Beal and Porter, 1991]. Standfast *et al.* [1979] found the overall seasonal pattern of SIDS in New York resembled that of viral respiratory infections.

**Table 1.1 The incidence of SIDS**

| <u>Country</u>           | <u>No/1000 live births</u> | <u>Reference</u>                       |
|--------------------------|----------------------------|--|
| <u>England and Wales</u> | 1.7                        | [Wigfield <i>et al.</i> , 1992]        |
| <u>Scotland</u>          | 1.33                       | [Registrar General for Scotland, 1992] |
| <u>Denmark</u>           | 1.9                        | [Helweg-Larsen., <i>et al.</i> , 1985] |
| <u>Australia</u>         |                            |  |
| Queensland               | 1.6                        | [SIDS by states and territories, 1988] |
| Tasmania                 | 3.5                        | [SIDS by states and territories, 1988] |
| <u>New Zealand</u>       |                            |  |
| Caucasians               | 3.9                        | [Borman <i>et al.</i> , 1988]          |
| Moaris                   | 6.5                        | [Borman <i>et al.</i> , 1988]          |
| <u>United States</u>     |                            |  |
| Native Indian            | 7.0                        | [Irwin <i>et al.</i> , 1992]           |
| White American           | 2.2                        | [Irwin <i>et al.</i> , 1992]           |
| Black American           | 3.75                       | [Blok, 1978]                           |
| <u>Hong Kong</u>         | 0.3                        | [Lee <i>et al.</i> , 1989]             |

## **1.2.2 Maternal factors**

Mothers' smoking has been identified as a risk factor for SIDS [Gibson, 1992; Scragg *et al.*, 1993]. Children of mothers who smoke have more respiratory tract infections [Bonham and Wilson, 1981]; and smokers are more likely to carry pathogenic bacteria such as meningococci [Blackwell *et al.*, 1990; Blackwell *et al.*, 1992] or staphylococci [Musher and Fainstein, 1981].

Rajegowda *et al.*[1978] found the incidence rate of SIDS of narcotic-dependent mothers was 5.5 fold higher than for all the babies born during the same time and in the same place. The intrauterine exposure to narcotics and its subsequent effect on central control of respiration in the young infant might be one of the underlying mechanisms for drug-related cases of SIDS. Other maternal factors associated with SIDS are: unmarried mother; young mother; high parity order; short inter-pregnancy interval; low birth weight; twins and poor attendance at antenatal clinics [Golding *et al.*, 1985].

## **1.2.3 Social factors**

There is a relationship between poor social conditions, increased morbidity, and SIDS [Biering-Sorensen, 1979]. Two factors associated with lower socioeconomic conditions are a higher proportion of women who smoke [Wald *et al.*, 1988] and the lower proportion who breast feed. Like respiratory and gastrointestinal illnesses, SIDS is reported to be associated more frequently with bottle feeding [Arnon, 1984].

## **1.3 Pathological findings in SIDS**

Although the diagnosis of SIDS is to some extent one of exclusion, there are a number of typical findings which are of value in diagnosis [Berry and Keeling, 1989]. Petechiae on the face are very rare in SIDS and raise the question of accidental suffocation.

Frothy fluid escaping from the nose and mouth is seen in more than half of SIDS victims and evidence of sweat in clothing suggests overheating [Fleming *et al.*, 1990]. Internal examination shows petechial haemorrhages in the thymus in a majority of cases and prominent lymph nodes. The lungs are usually well inflated. Minor inflammation and infection of the respiratory tract is common in SIDS. Mild fatty change in the liver is very common and is probably a non-specific marker of metabolic disturbances which accompany many types of illness or stress [Sinclair-Smith *et al.*, 1976; Bonnell and Beckwith, 1986]. The abnormal persistence of fetal haemoglobin and raised hypoxanthine levels in the vitreous humor might be due to periods of premortem hypoxia [Giulian *et al.*, 1987]. There is often gliosis in the brain stem [Becker, 1983] which might be the result of hypoxia or low grade inflammation. There is a delay in myelination and maturation of dendritic spines [Kinney *et al.*, 1991], possibly due to the effect of passive exposure to cigarette smoke.

## **1.4 Theories proposed for the aetiology of SIDS**

Epidemiological studies have led to several hypotheses; however, none can adequately explain all cases or all the features identified in the surveys.

### **1.4.1 Sleeping position**

There is a fear that babies sleeping supine are at increased risk of aspiration of vomitus, although this has not been established. Studies which looked at sleeping position found that the prone position was more common in babies dying of SIDS than in control babies [Beal and Finch, 1991]. The low incidence of SIDS in Hong Kong, which has a hot, humid climate and where infants sleep supine, has led to the suggestion that the prone position could be a risk factor [Lee *et al.*, 1989]. Studies in the Netherlands [Engelbert and de Jonge, 1990], New Zealand [Cowan, 1991] and in Avon [Wigfield

*et al.*, 1992] have shown a decline in the number of the infants dying from SIDS, and this decrease has been attributed to the campaign to discourage the prone sleeping position. These findings need more extensive examination of non-SIDS post-neonatal infant mortality rates in order to show that the decline in SIDS rate is not related to other factors. The SIDS rate in Scotland has fallen from 2.2 per 1000 in 1989 to 1.3 per 1000 in 1991 [Gibson *et al.*, 1991], but there were no changes in infant care specifically recommended in this population.

### **1.4.2 Clothing and overheating**

Room temperature, excess clothing and bedding are among the factors that could result in overheating of an infant. Overinsulation has been suggested to be a risk factor for SIDS. Fleming *et al.* [1990] showed that babies over 70 days of age who were wrapped in clothing and bedding in excess of 10 togs were 12 times more likely to die than babies wrapped in clothing and bedding less than 6 togs. The prone sleeping position will reduce heat loss by reducing the body surface area available for heat loss. In a heavily wrapped baby, the head, particularly the face, becomes the main route for heat loss, up to 85% of heat loss in the infant in bed [Wailoo, 1989]. When thick clothing and bedding are used, the thermoregulatory mechanisms could be compromised by the prone sleeping position [Nelson *et al.*, 1989].

The infant is vulnerable to changes in brain temperature. In animal studies small changes in hypothalamic temperature have profound effects on the control of respiration [Parmeggiani, 1987]. Infection increases the infant's basic metabolic rate and increases heat generation which could result in a significant change in thermal balance. In the Avon studies overwrapping in the presence of a viral infection increased the risk of SIDS [Gilbert *et al.*, 1992]. There is evidence that heat stress may be associated



with SIDS [Stanton, 1984; Sunderland and Emery, 1981] or with marked hypoventilation [Gozal *et al.*, 1988].

### **1.4.3 Apnoea and respiratory failure**

It has been suggested that healthy babies with viral infections often suffer from central apnoea, and babies with nasopharyngitis were more likely to become apnoeic [Steinschneider, 1975]. Prolonged apnoea and airway obstruction could be part of the pathophysiological process causing SIDS. Steinschneider *et al.* [1982] suggested that unstable respiratory activity during sleep as well as pharyngeal / laryngeal dysfunction might be induced by liquid stimulation. Southhall *et al.* [1990] recorded severe arterial hypoxaemia and intrapulmonary shunting in young children with recurrent cyanotic episodes. The most common stimulus was pain, fear or anger, and the onset of severe cyanosis was rapid with rapid loss of consciousness. Narrowing of the upper airways could be caused by respiratory infection and also lead to obstructive or possibly central apnoea [Tonkin *et al.*, 1979; Guilleminault, 1987; Guntheroth, 1989].

A study on the ventilatory response to hypoxia and hypercapnia in subsequent siblings of SIDS victims found that after 5 weeks of age, siblings of SIDS had a normal response to hypercapnia; however they responded to mild hypoxia with periodic breathing. Arousal occurred during 25% of the hypoxic challenges in the controls but was not seen in the siblings of SIDS victims [Brady and McCann, 1985]. This instability in ventilation might lead to severe apnoea and sudden death in the presence of other factors.

### **1.4.4 Cardiovascular abnormalities**

Congenital malformation of the conduction system of the heart might lead to sudden death from asystole or ventricular fibrillation [Southall, 1983]. Babies with this

malformation have prolonged Q-T intervals, a condition not easy to identify by morbid anatomical techniques. Schwartz [1976] suggested the cause of some SIDS could be sudden increases in sympathetic activity leading to imbalanced cardiac innervation. On the other hand, Steinschneider [1978] showed that the Q-T intervals in the siblings of victims did not differ from those of control infants, nor did the Q-T interval of parents. Kelly *et al.* [1977] concluded that prolonged Q-T interval does not play a major role in the genesis of either near-miss episodes or sudden infant deaths. Both heart rate and respiratory rate were greater in the preterm babies than in the term infants throughout the first six months of life; and, according to this concept, sudden infant death is linked to a vulnerable phase of cardiorespiratory maturation [Katona *et al.*, 1978]. Recently a study by Meny *et al.* [1994] has shown that bradycardia was an important feature in SIDS.

#### **1.4.5 Metabolic disorders**

A small percentage of babies who die from SIDS have an inborn error of metabolism [Emery *et al.*, 1988]. An abnormality of fatty acid  $\beta$ -oxidation, medium chain acyl coenzyme-A dehydrogenase (MCAD) deficiency, could be responsible for 3% of cases of SIDS [Howat *et al.*, 1984]. This possibility needs to be considered in cases showing micro-vesicular fatty changes in the liver and other organs [Howat *et al.*, 1986]. The enzyme is important in the metabolism of fatty acids in the liver and plays an essential part in maintaining energy homeostasis during fasting. Asymptomatic presentations were reported in 12% of MCAD deficiency victims [Touma and Charpentier, 1992]; otherwise, in MCAD, the sufferers present characteristically with vomiting, lethargy and hypoglycaemia that is provoked by fasting [Roe and Coates, 1989]. In a recent study by Miller *et al.* [1992] MCAD deficiency did not make a significant contribution to the aetiology of SIDS. A study by Burchell *et al.* [1989] has linked the deficiency in

glucose-6 phosphatase enzyme which is an inborn error of metabolism with few cases of SIDS.

#### **1.4.6 Immunological abnormalities in SIDS**

Lymphoid tissue in the lungs and spleen of SIDS victims can be more prominent than in controls suggesting prior episodes of infection [Emery and Dinsdale, 1974; Barzanji *et al.*, 1976]. Many studies have failed to demonstrate a consistent abnormality in humoral immunity [Valdes-Dapena, 1982]. Recently Stoltenberg *et al.* [1992] reported evidence that the mucosal immune system is stimulated in SIDS. They found higher numbers of IgM immunocytes in the tracheal wall and significantly higher IgA cells in the duodenal mucosa of SIDS victims compared with a group of infants that died from non-infectious causes.

Anaphylaxis to cows' milk has been suggested as a cause of some cases of SIDS [Coombs and McLaughlan, 1982] but there was neither a consistent association of SIDS with atopy [Warnasuriya *et al.*, 1980] nor an association of an increase in total serum IgE with milk or in allergen-specific IgE [Mirchandani *et al.*, 1984; Clark *et al.*, 1979]. An increase in specific IgE against a fungus *Aspergillus fumigatus* and a house dust mite *Dermatophagoides pteronyssinus* has been reported [Turner *et al.*, 1975]. Howat *et al.* [1994] found that the victims of SIDS have three times more eosinophils in their lungs, accompanied by increased T lymphocytes and B lymphocytes and there were more peribronchial mast cells compared to those for controls. Eosinophils can produce the potent pyrogenic cytokines TNF- $\alpha$  and macrophage inflammatory protein-1 $\alpha$  [Costa *et al.*, 1993] which could cause life-threatening hyperpyrexia. In addition, they can secrete both oxygen free radicals and cytotoxic proteins [Frigas and Gleich, 1986] which could lead to pulmonary oedema.

Mast cells are important factors in immediate hypersensitivity reaction [Holgate and Church, 1992]. There are some indications for mast cell degranulation in SIDS [Harrison *et al.*, 1993; Walls *et al.*, 1993]. The latter team found an increase in serum tryptase which is a marker for the degranulation.

### **1.4.7 Infectious agents**

Infants are particularly vulnerable to infection during the period when most cases of SIDS occur (2-4 months). This is when maternal antibodies have declined and the infant immune system is immature.

#### **1.4.7.1 Association of viruses with SIDS**

The findings that many SIDS infants experienced minor respiratory illness before death suggested these deaths might be caused by viruses. In areas with a temperate climate, cot deaths occur most often in the winter months during which epidemics of respiratory virus infection are common. Epidemiological studies provided inconsistent evidence that respiratory viruses are involved in SIDS, and different research workers have drawn different conclusions concerning the significance of viruses in the aetiology of SIDS. Gilbert *et al.* [1992] pointed out that viral infection alone was not a major risk factor as long as babies were lightly wrapped; but, in the heavily wrapped babies, the presence of a viral infection greatly increased the risk of death.

The technical problems associated with identification of viruses probably resulted in the underestimation of their presence among SIDS cases in early studies [Fleming, 1992]. A recent study in which the polymerase chain reaction (PCR) technique was used to detect viral nucleic acids in tissues from SIDS infants and infants who died of known causes, found a higher proportion of SIDS infants had evidence of virus

infection, mainly adenoviruses [An *et al.*, 1993]. Other viruses identified among SIDS infants include: influenza virus [Nelson *et al.*, 1975; Zink *et al.*, 1987]; enterovirus [Danes, 1989]; rhinovirus [Las Heras *et al.*, 1983]; and rotavirus [Yolken and Murphy, 1982].

In two out of three studies, there was an association between the peak of SIDS and total viral respiratory isolates in the community [Carpenter and Gardner, 1990; Uren *et al.*, 1980]. A survey of SIDS cases and controls matched for age, time and geographical area found no differences in isolation of viruses [Gilbert *et al.*, 1992]. Only a weak association of viruses with SIDS has been reported in a New Zealand survey [Ford *et al.*, 1990].

One of the viruses commonly associated with SIDS is respiratory syncytial virus (RSV) [Ogra *et al.*, 1975; Williams *et al.*, 1984]. In a major Australian study, 90% of RSV isolates were from SIDS infants older than 3 months [Williams *et al.*, 1984]. It has been suggested that viral infection might be more important in those SIDS cases aged over 3 months in which evidence of respiratory tract inflammation has been found at autopsy [Williams, 1980].

#### **1.4.7.2 Association of toxigenic bacteria with SIDS**

There has been no substantial evidence for invasive bacterial infections in SIDS [Banks, 1958 and Valdes-Dapena, 1967]; therefore much of the work on the role of bacteria in SIDS has concentrated on species that produce exotoxins (Table 1.2).

A characteristic feature of SIDS is the age distribution. In a hypothesis paper by Morris *et al.* [1987] the age distribution of SIDS which rises from birth to a peak at 4 months and is followed by a progressive fall matched the predicted age distribution

**Table 1.2 Toxin producing bacteria implicated in SIDS.**

| <u>Species</u>               | <u>Toxin</u>           |
|------------------------------|------------------------|
| <i>Staphylococcus aureus</i> | A, B, C, D and TSST-1. |
| <i>Bordetella pertussis</i>  | pertussis toxin        |
| <i>Escherichia coli</i>      | enterotoxin            |
| <i>Clostridium botulinum</i> | A, B, C, F and G       |
| <i>Clostridium difficile</i> | A and B                |

curves for susceptibility to bacterial toxins. At the time maternal antibodies are declining, infants will be vulnerable to bacterial toxins produced by the normal microbial flora.

Brandt [1970] found significant bacterial isolates in the lung tissue or blood of 16% of SIDS victims and Telford *et al.* [1989] isolated *S. aureus*, *Streptococcus viridans* and *Escherichia coli* more commonly from 46 SIDS victims than from healthy live babies. A significantly higher proportion of toxigenic bacteria and their toxins were found in faecal samples of SIDS babies than samples from the comparison group, these toxins were also found in serum from the SIDS babies [Murrell *et al.*, 1993].

#### **1.4.7.2.1 Toxigenic bacteria in the intestinal tract**

Infant botulism can account for some unexpected infant deaths. *C. botulinum* toxin was found in the intestines of 4% of cot deaths in the United States [Arnon *et al.*, 1987] and up to 16% of those investigated in Sweden [Sonnabend *et al.*, 1985]. There is little evidence for this in the United Kingdom [Turner *et al.*, 1978].

In an experimental model, toxins A and B from *C. difficile* produced a rapid death in the infant rhesus monkey [Arnon *et al.*, 1984], but there is little evidence to suggest that *C. difficile* is more common among SIDS infants [Gurwith *et al.*, 1981; Cooperstock *et al.*, 1982]. *C. perfringens* enterotoxin A has been identified in gastrointestinal contents and sera of some SIDS infants, but it is not clear if the toxin is responsible for these deaths [Murrell *et al.*, 1987]. Toxigenic *E. coli* were also isolated in a greater number of SIDS victims compared to normal control infants [Bettleheim *et al.*, 1989; Bettleheim *et al.*, 1990].



#### **1.4.7.2.2 Toxigenic bacteria in the respiratory tract**

Two toxigenic bacteria that colonize the respiratory tract have been suggested to be involved in SIDS, *Bordetella pertussis* [Nichol and Gardner, 1988] and *S. aureus* [Morris *et al.*, 1987].

#### ***B. pertussis* and SIDS**

Although there are no reports that *B. pertussis* has been isolated from SIDS infants, they are difficult to culture even from children with symptomatic whooping cough [Davis *et al.*, 1990]. A study by Thomas and Lombart [1987] reported that the rate of isolation of *B. pertussis* was 35% among individuals with typical disease manifestations of pertussis while the rate was only 4% among those with asymptomatic disease. Young infants with pertussis do not whoop, but can have expiratory apnoea and there is an excess of unexplained infant deaths during epidemics of whooping cough [Nicol and Gardner, 1988]. There is a suggestion that among infants not immunised against diphtheria, pertussis and tetanus (DPT) there is increased risk of infection which could lead to hypoxaemia [Southall *et al.*, 1988].

*B. pertussis* produces several toxins which contribute to the pathophysiology of the striking effects caused by these bacteria. Among these toxins are pertussis toxins and filamentous haemagglutinin. Both of these toxins are expressed on the surface of *B. pertussis* [Tuomanen and Weiss, 1985; Cowell *et al.*, 1986]. The absence of either of these components greatly diminished the ability of the bacteria to attach to human ciliated respiratory cells [Tuomanen *et al.*, 1985]. Pertussis toxin is also known as lymphocytosis promoting factor, histamine-sensitising factor, or islet-activating protein. It is an A-B toxin: B is the binding oligomer and the A is the enzymatically active part. It has been reported that Le<sup>a</sup> and Le<sup>x</sup> are receptors for the toxin [van



t'Wout *et al.*, 1992]. Pertussis toxin acts by ADP ribosylating the GTP-binding protein of the mammalian membrane [Ui, 1988]. Its main activities in the mouse are: an adjuvant for IgE antibodies; sensitisation to histamine and serotonin; enhancement of insulin secretion; and induction of leukocytosis and lymphocytosis [Munoz, 1985]. The cause of death in some cases of SIDS could be hypoglycaemia via the toxin's effect on the islets. Pertussis toxin enhances hypersensitivity reactions, and degranulation of mast cells have been observed in autopsy material from SIDS infants [Harrison *et al.*, 1993; Howat *et al.*, 1994].

## ***S. aureus* and SIDS**

There is indirect evidence that nasopharyngeal colonization by toxigenic strains of *S. aureus*, particularly those producing the toxic shock syndrome toxin-1 (TSST-1), might be associated with some of these infant deaths [Morris *et al.*, 1987]. Compared with healthy live infants (28.3%), *S. aureus* was isolated from a higher proportion of SIDS infants (41.3%) [Telford *et al.*, 1989]. Among the *S. aureus* strains 30-50% are toxigenic [Mackie and McCartney, 1989]. Recently, a study by Murrell *et al.* [1993] found 66.7% of the *S. aureus* isolated from faecal samples of SIDS cases were enterotoxigenic ones compared to 85.7% non-toxigenic among the isolates from healthy babies. One study has identified the staphylococcal enterotoxin C in the kidneys of 36% of SIDS infants compared with 12% of non-SIDS infants [Malam *et al.*, 1992]. TSST-1 was detected by immunohistochemical techniques in 9 of 50 kidneys from SIDS infants compared with 3 of 50 control necropsy kidneys [Newbould *et al.*, 1989].

*S. aureus* is a versatile human pathogen capable of causing a variety of diseases, ranging from minor skin infections to life-threatening conditions such as toxic shock syndrome. Its versatility is mirrored in the range of virulence factors that it is capable

of producing. Among these factors are a group of enterotoxins: A, B, C<sub>1-3</sub>, D, E, staphylococcal exfoliative toxin, toxic shock syndrome toxin-1 (TSST-1). The term 'superantigens' has been suggested for these proteins and other proteins that have in common an extremely potent stimulatory activity for T lymphocytes. The process of stimulation of T-cells leads to production of cytokines by T-cells and monocytes [Mosmann and Coffman, 1989]. *In vitro* stimulation of human or murine cells with these toxins induced interferon- $\gamma$ , interleukin-2 (IL-2) and tumour necrosis factor (TNF) [De Azavedo *et al.*, 1988; Micusan *et al.*, 1989]. The levels of IL-2, IL-6 and TNF in the serum of patients with meningococcal septic shock correlated with fatal shock [Waage *et al.*, 1989].

The pyrogenic staphylococcal toxins and streptococcal pyrogenic exotoxins are powerful substances which can kill previously healthy adults [Bohach *et al.*, 1990]. They are potent inducers of fever ( $> 38.5^{\circ}\text{C}$ ) and might account for the high temperatures recorded for SIDS infants at the time of autopsy [Sunderland and Emery, 1981]. Both Interleukin-1(IL-1) and TNF are thought to induce fever by the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in the preoptic area of the hypothalamus [Dinarello and Wolff, 1982; Dinarello *et al.*, 1986]. IL-1 has been suggested to cause deep sleep associated with prolonged periods of apnoea [Guntheroth, 1989], pyrogenic toxins enhance endotoxic shock and also cause enhancement of delayed type hypersensitivity [Schlievert *et al.*, 1979].

#### **1.4.7.3 Synergy between toxins and environmental factors**

There is no consistent evidence for either temporal or geographical clusters of SIDS [Golding *et al.*, 1985]. This suggests that a single infectious agent is probably not responsible for SIDS; however, synergistic effects between infectious agents or toxins and environmental factors might be involved in SIDS. In experimental models, synergy

occurs between toxins of the same microorganism or between toxins of two different microorganisms, as in the case of an enhanced lethality of staphylococcal toxin in the presence of preparations from *E. coli* in the chick embryo model [Drucker *et al.*, 1992]. Similarly, *S. aureus* TSST-1 and endotoxin showed enhanced toxicity on chicken embryos when both toxins were combined [de Azavedo *et al.*, 1985].

Synergy between bacterial toxins (staphylococcal  $\alpha$  and  $\gamma$  toxins, endotoxins and diphtheria toxin) and influenza virus infection have been observed in the infant ferret model [Jakeman *et al.*, 1991; Smith and Sweet, 1988]. In clinical medicine, toxic shock syndrome caused by toxin producing staphylococci has been identified as a complication of influenza or influenza-like illness [McDonald *et al.*, 1987], and endotoxin is found in the blood of patients with toxic shock syndrome [Stone and Schlievert, 1987].

Mothers' smoking was identified as a factor for SIDS in the New Zealand studies [Mitchell, 1991; Scragg *et al.*, 1993]. Children in households where there are smokers have more respiratory tract infections [Bonham and Wilson, 1981]. Among studies of exposure to cigarette smoke and infection, as a rule, the strongest associations are found with smoking habits of the mother [Pershagen, 1986].

Smokers are also more likely to carry potentially pathogenic bacteria in the throat, *e.g.*, meningococci [Blackwell *et al.*, 1990; 1992] and staphylococci [Musher and Fainstein, 1981]. The prolonged intimate contact between mother and infant could result in greater risk of exposure to potentially pathogenic bacteria resident in the mother's nasopharynx.

Smoking and passive exposure to cigarette smoke might also increase susceptibility to respiratory viral infection and subsequent colonization by staphylococci [Ramirez-ronda *et al.*, 1981].

## **1.5 Blood group antigens and susceptibility to infectious agents**

The first significant associations between blood group phenotypes and human diseases were noted in 1953 by the discovery of an association between blood group A and carcinoma of the stomach [Aird *et al.*, 1953]. The reports of associations between human disease and ABO blood groups and the ABH secretor system were collected and reviewed by Mourant *et al.* [1978]. Non-secretors were over-represented among patients with rheumatic fever and also among asymptomatic carriers of group A *Streptococcus pyogenes* [Haverkorn and Goslings, 1969]. Subsequent epidemiological studies demonstrated increased susceptibility of non-secretors of ABH blood group antigens to infections due to some bacteria and yeast [Blackwell *et al.*, 1989].

This section is organized to provide: a brief review of blood groups and disease susceptibility; a review of the structures, genetics and control of expression of blood group antigens in relation to secretor status; and the hypotheses proposed to explain the epidemiological associations between blood groups and secretor status and susceptibility to infectious agents.

### **1.5.1 Summary of epidemiological studies**

Epidemiological studies have found associations between infectious diseases and ABO blood group antigens (Table 1.3). Evidence for associations between non-secretion of ABO blood group antigens and susceptibility to bacterial and fungal infection have

**Table 1.3 Association between ABO and infectious diseases.**

| <u>Infectious agent</u>              | <u>ABO association</u> |
|--------------------------------------|------------------------|
| <u>Oral cavity</u>                   |                        |
| <i>C. albicans</i> (carriage)        | O                      |
| Periodontal disease                  | O and AB               |
| <u>Respiratory tract</u>             |                        |
| Influenza A                          | O and B                |
| Influenza A <sub>2</sub>             | O                      |
| <i>Strept. pyogenes</i> Group A      | not O                  |
| <i>Strept. pneumoniae</i>            | not B                  |
| <i>M. tuberculosis</i>               | O                      |
| <u>Gastrointestinal tract</u>        |                        |
| <i>E. coli</i>                       | B                      |
| <i>Salmonella</i> and <i>E. coli</i> | B and AB               |
| <i>V. cholerae</i>                   | O                      |
| <u>Urinary tract</u>                 |                        |
| <i>E. coli</i>                       | B                      |
|                                      | B/AB                   |
| <u>Genitourinary tract</u>           |                        |
| <i>N. gonorrhoeae</i>                | B                      |
| Malaria                              | A                      |

Table modified from Blackwell [1989].

been noted. In contrast, studies of secretor status and viral infection indicate secretors are at greater risk of infection (Table 1.4). Also an association was observed between non-secretion and some autoimmune diseases for which infectious aetiologies have been proposed; among patients with spondyloarthropathies [Shinebaum *et al.*, 1987], insulin dependent diabetes mellitus [Blackwell *et al.*, 1987] and Graves' disease [Collier *et al.*, 1988].

### **1.5.2 ABO blood group antigens**

The ABO blood group system in humans was first recognised by Landsteiner in 1901. By mixing separated sera with suspensions of red cells obtained from different individuals, a pattern of agglutination reactions was observed; and from these observations, four major blood groups were identified (Table 1.5).

The frequencies of the four ABO groups vary in different populations [reviewed by Race and Sanger, 1975]. In the United Kingdom the percentage for each group is: A (42%); B (8.5%); AB (3%); O (46.5) [Mollison *et al.*, 1987]. American Indians are almost exclusively group O while Asians have a higher incidence of group B.

Only man and the great apes have red cells expressing ABH antigens. The A and B blood group antigens are major histocompatibility antigens [Joysey *et al.*, 1977; Hershko *et al.*, 1980] and their incompatibility is a cause of graft and transplant rejection for many tissues and organs. The antigens of the ABO blood groups appear to be absent from the central nervous system.

**Table 1.4 Infectious diseases and secretor-status**

| <b><u>Infectious microorganism</u></b>       | <b><u>Reference</u></b>  |
|--|--|
| <b><u>Non-secretors</u></b>                  |  |
| <b><u>Oral cavity</u></b>                    |  |
| <i>C. albicans</i> (infections and carriage) | Aly <i>et al.</i> , 1991; 1992; Thom <i>et al.</i> , 1989;<br>Burford-Mason <i>et al.</i> , 1988 |
| Caries                                       | Holbrook and Blackwell, 1989   |
| <b><u>Respiratory tract</u></b>              |  |
| <i>Strep. pyogenes</i>                       | Haverkorn and Goslings, 1969   |
| <i>Strep. pneumonia</i>                      | Blackwell <i>et al.</i> , 1986a  |
| <i>N. meningitidis</i>                       | Blackwell <i>et al.</i> , 1986a  |
| <i>H. influenza</i> (type b)                 | Blackwell <i>et al.</i> , 1986b  |
| <b><u>Gastrointestinal tract</u></b>         |  |
| <i>V. cholerae</i>                           | Chaudhuri and Das Adhikary, 1978   |
| <b><u>Genital tract</u></b>                  |  |
| <i>C. albicans</i>                           | Thom <i>et al.</i> , 1989  |
| <b><u>Secretors</u></b>                      |  |
| RSV  | Raza <i>et al.</i> , 1991  |
| Influenza A and B                            | //   |
| Echo virus                                   | //   |
| Rhino virus                                  | //   |
| HIV  | Blackwell <i>et al.</i> , 1991   |

**Table 1.5    Antigens and isohaemagglutinins of the ABO blood group system**

| <b><u>Group</u></b> | <b><u>Antigens on red cells</u></b> | <b><u>Antibodies in serum</u></b> |
|---------------------|-------------------------------------|-----------------------------------|
| A                   | A, H*                               | Anti-B                            |
| B                   | B, H*                               | Anti-A                            |
| AB                  | A, B, H*                            | None                              |
| O                   | H*                                  | Anti-A and Anti-B                 |

\* H is the antigen found on group O cells; H is also present on cells of the other 3 groups.



New-born infants do not produce isohaemagglutinins until 3-6 months of age. The naturally occurring antibodies of the majority of group A or B individuals are mainly IgM and produced in response to A and B-like antigens in the environment, such as those microbes in the gut and respiratory tract. A and B-like antigens have been detected in a variety of microorganisms and animal tissues [Springer, 1956; Springer, 1965]; and it is suggested these antigens induce isohaemagglutinins in humans.

### **1.5.3 Genetics of the ABO blood groups and secretor status**

The *genes* controlling the expression of A or B antigens are autosomal dominant alleles present on chromosome 9 [Westerveld *et al.*, 1976]. Group O individuals possess a recessive allele at the ABO locus on both homologous chromosomes. The O allele is thought to code for a functionally inert protein incapable of modifying the H antigen [Yamamoto *et al.*, 1990] while A and B genes code for a N-acetylgalactosamine- and galactosyl-transferase respectively.

ABH antigens can be detected in most secretions and tissues of the human body [Clausen and Hakomori, 1989]. They can be detected in the semen and the saliva of the majority of individuals. These individuals are called secretors while those in whom ABO antigens are not found in body fluids are called non-secretors. The ability to secrete the ABO blood group antigens into body fluids is controlled by the secretor (*Se*) gene which is located on chromosome 19 [Watkins, 1980]. The *Se* gene is inherited in a Mendelian dominant pattern; therefore, secretors are either *Se/Se* or *Se/se* and their predicted proportion within a population is 75-80%. Non-secretors are *se/se* and represent about 20-25% of the population [Race and Sanger, 1975]. This ratio can vary widely in different ethnic groups and some geographically isolated populations [Mourant *et al.*, 1978; Eriksson *et al.*, 1986].

The phenotype O is thought to be due to a gene that does not produce a gene product since no antibody was found that reacted with O cells but did not react with cells bearing the A or B antigens. An extract of the European gorse bush *Ulex europaeus* agglutinates blood cells of different types and it is suggested that this reagent identified an antigen on the surface of the red cell that is characteristic of type O red cells. This antigen is called the H antigen.

The antigen H is present on red cells of nearly all humans. The *H* and *Se* genes are closely linked on the short arm of chromosome 19 [Oriol *et al.*, 1986]. Le Pendu [1983] found two different forms of fucosyl transferases associated with the *H* and *Se* genes. Bombay individuals have the *h/h*, *se/se* genotype and fail to express ABH antigens in cells or secretions [Oriol *et al.*, 1981]; whereas individuals with the para-Bombay phenotype have *h/h*, *Se/Se* or *h/h*, *Se/se*. They will express type 1 precursor and products derived from it in their secretions but will have no ABH antigens on their red cells.

#### **1.5.4 Structure of the ABH antigens**

The first insight into the structures of these antigens came from the work of Morgan and Watkins. A, B and H substances are found as glycolipids, mainly present in the cell membranes, and as glycoproteins, mainly present in secretions. A, B, or H antigens are found on a variety of precursor molecules. There are 4 main types of these precursor molecules (Table 1.6) to which different monosaccharides are added by glycosyl transferases to produce the A, B and H antigens. Type 1 chains are found mainly in secretions. Type 2 chains [Oriol *et al.*, 1986] are found on the red cells as well as most other cells of the body except the brain. Not much information is available with regard to type 3 and 4 precursor chains [Clausen *et al.*, 1986].

On red cells, the H type 2 antigen is produced by the transfer of a fucose molecule from guanosine diphosphate L-fucose to the terminal galactose molecule at the C2 position by the  $\alpha$ -1 $\rightarrow$ 2 fucosyltransferase encoded by the *H* gene (Figure 1.1). The A and B antigens are formed from the H antigen [Painter *et al.*, 1965; Lloyd *et al.*, 1966]. For individuals with the A gene, the glycosyltransferase adds an N-acetylgalactosamine (GalNac) residue from uridine diphosphate N-acetylgalactosamine in an  $\alpha$ 1-3 bond to the H antigen. The *B* gene codes for an enzyme that transfers a galactose (Gal) residue from uridine diphosphate D-galactose to the same acceptor. For AB individuals some chains will carry the A determinant and others will have the B determinant [Watkins, 1967]. These enzymes can glycosylate both simple, straight chain, and complex molecules provided that the terminal residues form the H antigen.

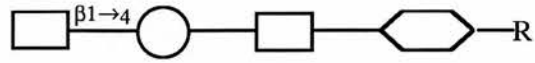
In secretions, the type 1 chain is fucosylated by  $\alpha$ 1-2 fucosyl transferase coded for by the secretor gene to produce H type 1 [Watkins *et al.*, 1988]. H type 1 acts as a substrate for A or B glycosyltransferase to form A or B determinants (Figure 1.2)

**Table 1.6 Structures of precursor chains**

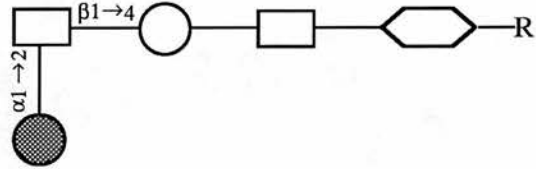
|   |                  |
|---|------------------|
| <b>Gal<math>\beta</math>1<math>\rightarrow</math>3GlcNac<math>\beta</math>1<math>\rightarrow</math>R</b>  | Precursor type-1 |
| <b>Gal<math>\beta</math>1<math>\rightarrow</math>4GlcNac<math>\beta</math>1<math>\rightarrow</math>R</b>  | Precursor type-2 |
| <b>Gal<math>\beta</math>1<math>\rightarrow</math>3GalNac<math>\alpha</math>1<math>\rightarrow</math>R</b> | Precursor type-3 |
| <b>Gal<math>\beta</math>1<math>\rightarrow</math>3GalNac<math>\beta</math>1<math>\rightarrow</math>R</b>  | Precursor type 4 |

(Gal: galactose; GlcNac: N-acetylglucosamine; R: Glycolipid / Glycoprotein)

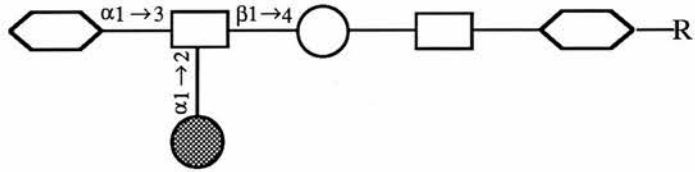
precursor type 2



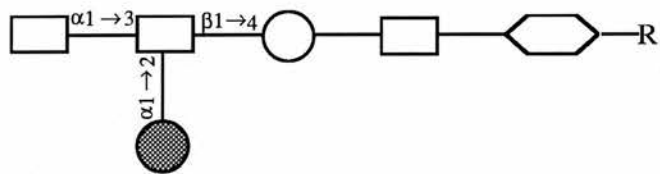
H type 2



A antigen



B antigen



D-galactose



N-acetyl-D-glucosamine



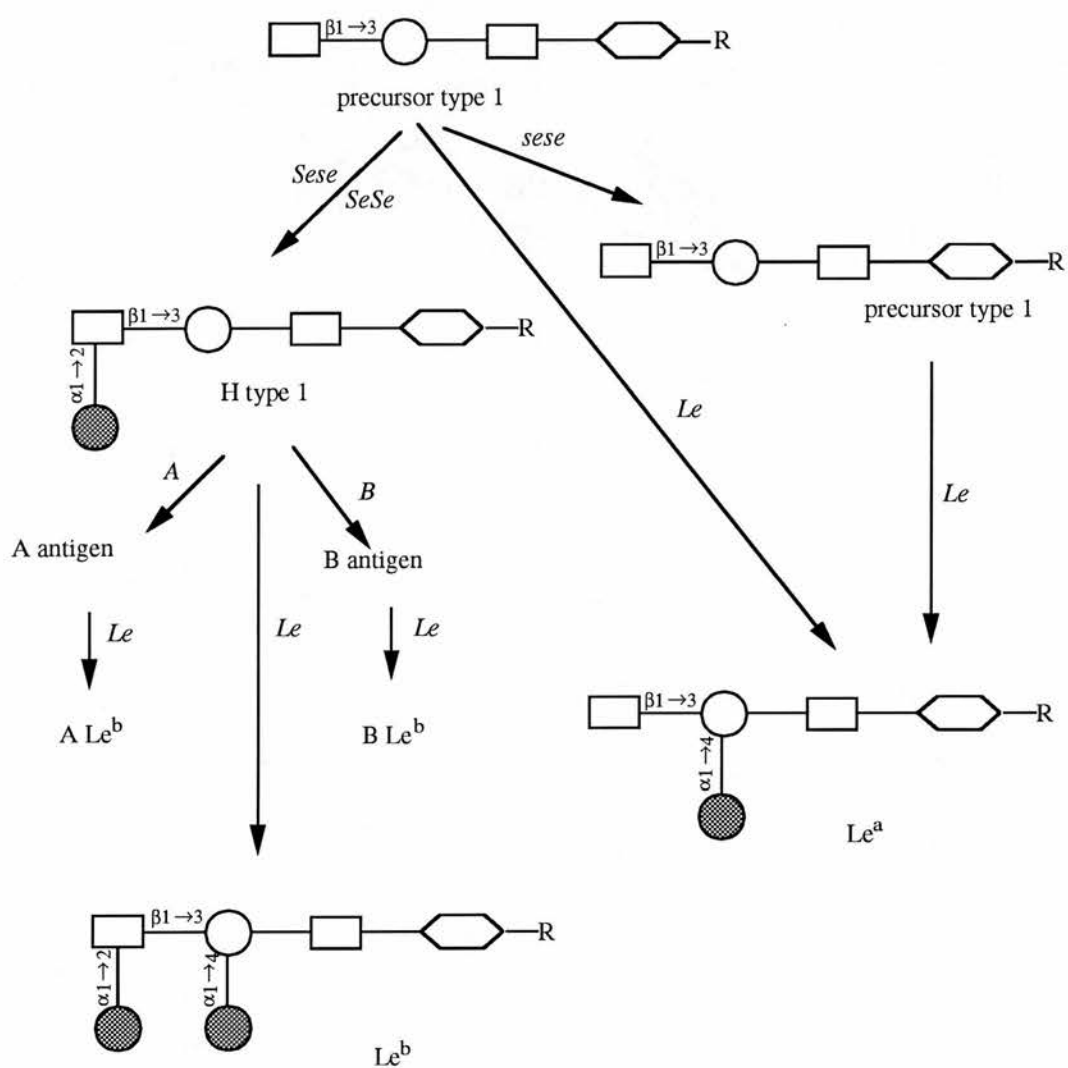
fucose



N-acetyl-D-galactosamine

R = glycolipid

**Figure 1.1 Schematic diagram of the H, A and B structures derived from precursor type 2**



**Figure 1.2** Formation of H, A, B and Lewis antigens from precursor type 1.

**R** = glycoprotein and other symbols as in Figure 1.1.

## 1.5.5 Lewis blood group antigens

In 1946, Mourant discovered an antibody that identified a new antigen. This antigen is called Lewis<sup>a</sup> (Le<sup>a</sup>). A few years later, Lewis<sup>b</sup> (Le<sup>b</sup>) was discovered by Anderson [1949]. Le<sup>a</sup> and Le<sup>b</sup> antigens do not form part of the structure of the cell membrane and are not synthesized on the red cells but are present in the plasma and in the body fluids such as saliva and ovarian cyst fluid. In secretions, the Lewis determinants are carried on the same glycoprotein molecules as the ABH antigens [Watkins, 1974]. The frequencies of the Lewis red cell phenotypes in the United States when tested with anti-Le<sup>a</sup> and anti-Le<sup>b</sup> are shown in Table 1.7 [Issit, 1986]. This ratio can vary in different ethnic groups and some isolated populations. Almost all American Indians and Eskimos are secretors, and Icelanders have the highest proportion of non-secretors (42%) [Mourant *et al.*, 1976; Eriksson *et al.*, 1986].

### 1.5.5.1 Genetics and structure of the Lewis system

The Lewis gene is located on chromosome 19 but is independent of the *H* and *Se* loci [Elberg *et al.*, 1983]. The secretor gene contributes to expression of the Lewis blood group antigens. Non-secretor individuals produce only Le<sup>a</sup> in their secretions. Secretor individuals produce Le<sup>b</sup> predominantly and variable amounts of Le<sup>a</sup> in their secretions as well as ABH determinants on type 1 precursor chains [Race and Sanger, 1975]. Individuals who lack any Lewis determinants on their red blood cells are called Lewis negative. The distribution of the ABO and Lewis antigens on cells and in body fluids of secretors and non-secretors is summarized in Table 1.8

**Table 1.7** Frequencies of the Lewis phenotypes.

| <u>Phenotype</u>   | <u>Frequency in Caucasians</u> |
|--------------------|--------------------------------|
| Le <sup>a+b-</sup> | 22 %                           |
| Le <sup>a-b+</sup> | 72 %                           |
| Le <sup>a-b-</sup> | 6 %                            |

**Table 1.8 Blood group antigens on cells and in body fluids of secretors and non-secretors.**

| <u>Antigen</u>  | <u>Secretors</u> |                   | <u>Non-secretors</u> |                   |
|-----------------|------------------|-------------------|----------------------|-------------------|
|                 | <u>Cells</u>     | <u>Secretions</u> | <u>Cells</u>         | <u>Secretions</u> |
| H (A/B)         | +                | +                 | +                    | -                 |
| Le <sup>a</sup> | - (+)*           | - (+)*            | +                    | +                 |
| Le <sup>b</sup> | +                | +                 | -                    | -                 |

\* Present in variable quantities.



The Lewis gene codes for an  $\alpha$ 1-3/4 fucosyl transferase which adds L-fucose to N-acetyl-D-glucosamine of the type 1 precursor to form Le<sup>a</sup> or to the H type 1 to form Le<sup>b</sup> [Oriol *et al.*, 1986] (Fig 1.2). Lewis antigens are also found on type 2 precursors; Le<sup>x</sup> is the equivalent of Le<sup>a</sup> and Le<sup>y</sup> the equivalent of Le<sup>b</sup>. The *x* gene codes for an  $\alpha$  1-3 fucosyltransferase that adds fucose to the subterminal N-acetyl-glucosamine on the type 2 precursor.

#### **1.5.5.2 Interaction between ABO, H, Se and Le genes**

The genetic make up of the individual regulates the expression of the blood group determinants through a series of glycosyl transferase enzymes acting on precursor chains. The specificity of the glycosyl transferases will either render the end product acceptable for another enzyme or unusable by other enzymes as a substrate. The enzyme coded by the secretor gene adds fucose to the terminal sugar of the type 1 precursor and that coded for by the Lewis gene adds fucose to the subterminal sugar of the type 1 precursor. If the secretor enzyme adds fucose first, the structure can act as a substrate for the Lewis enzyme to form Le<sup>b</sup> antigen. If the Lewis enzyme adds fucose to the subterminal sugar first to form Le<sup>a</sup>, the secretor gene can not use the Le<sup>a</sup> as a substrate to form Le<sup>b</sup> [Watkins *et al.*, 1988] (figure 1.2).

In infants, the enzyme coded by the secretor gene does not appear to be as efficient as the one coded for by the Lewis gene. The result is that, even though they are genetically secretors, infants express considerable amounts of Lewis<sup>a</sup> on their cells. The relative amounts found will depend on the efficiency of the enzymes [Watkins *et al.*, 1988; Ogata *et al.*, 1988].

## **1.6 Hypotheses to explain the associations between susceptibility to infectious agents and blood group antigens and secretor status**

### **1.6.1 Isohaemagglutinins and immune responses**

Anti-A and anti-B isohaemagglutinins could act as natural bactericidal and opsonic antibodies for some strains of microorganisms [Check *et al.*, 1972; Muschel and Osawa, 1959]. Reed *et al.* [1974] reported a significant lower proportion of group B individuals among patients with pneumococcal disease and carriers of *Strep. pneumoniae*. They have shown that pneumococci could acquire either A or B antigens in the growth medium and with lysed bacteria they demonstrated an enzyme capable of altering B antigen to the A-like antigen. Some of the strains tested expressed antigens which cross reacted with A antigen. They suggested that anti-A isohaemagglutinins of B individuals might act as natural antibodies against the bacteria. In contrast, the anti-A and anti-B antibodies enhanced binding of *N. gonorrhoea* by human monocytes and those of group B bound more bacteria than monocytes of other ABO blood groups. This has been suggested to contribute to increased susceptibility of B blood group individuals to gonorrhoeae [Kinane *et al.*, 1983].

Lower levels of both salivary and serum IgA were reported for non-secretors [Waissbluth and Langman, 1971; Grunbacher, 1972]. A study by Blackwell [1989] did not confirm this observation as no difference in total serum or salivary IgA was found between secretors and non-secretors among carriers of *N. meningitidis*. Also there was no difference between secretors and non-secretors in spondloarthropathies [Shinebaum *et al.*, 1987]. In contrast, Zorgani *et al.* [1992] using the quantitative ELISA method

found significantly higher levels of total salivary IgM and specific IgM antibodies against *N. lactamica* and *N. meningitidis* among secretors compared with non-secretors.

### **1.6.2 The complement component 3 (C3) gene**

Both the gene that codes for C3 and the *Se* gene are in the same linkage group on chromosome 19 [Elberg *et al.*, 1983]. In a study by Blackwell *et al.* [1988a] the levels of C3 among non-secretors in non-carriers of *N. meningitidis* was found to be slightly lower than those for the secretors. Lower levels of C3 and C4 complement components were also reported among patients with insulin dependent diabetes [Charlesworth *et al.*, 1987], but not among sufferers of non-insulin diabetes [Charlesworth *et al.*, 1982]. A study by Blackwell *et al.* [1987] showed that there was a significant increase in the number of non-secretors among individuals with insulin dependent diabetes and there were lower levels of both C3 and C4 among non-secretors [Blackwell *et al.*, 1988b].

### **1.6.3 Blood group antigens: receptors for microorganisms**

There are several pathogens which use blood group antigens as receptors on host cells (Table 1.9). An association between the Duffy blood type and susceptibility to malaria due to *Plasmodium vivax* has been reported [Miller *et al.*, 1975]. Duffy antigen is expressed in white populations but is absent in the majority of African and American Blacks [Sanger *et al.*, 1955]. The Duffy antigen has been demonstrated in vitro to be a receptor for *P. knowlesi* which is closely related to *P. vivax* and this might explain why African Blacks are more resistant to malaria infection caused by *Plasmodium vivax* than the whites [Miller *et al.*, 1975]. Barnwell *et al.* [1989] have shown in vitro the Duffy antigen is a receptor for *P. vivax* and *P. knowlesi* merozoites. The P blood group

**Table 1.9 Microorganisms that use blood group antigens as receptors**

| <u>Microorganisms</u>               | <u>Blood Group receptor</u> | <u>Reference</u>                |
|-------------------------------------|-----------------------------|---------------------------------|
| <i>Candida</i>                      | Lewis <sup>a</sup>          | May <i>et al.</i> , 1989        |
| <i>Escherichia coli</i>             |                             |                                 |
| Uropathogenic strains               | P                           | Kallenius <i>et al.</i> , 1980  |
|                                     | M                           | Vaisanen <i>et al.</i> , 1982   |
|                                     | N                           | Jann <i>et al.</i> , 1988       |
| Septicaemia and neonatal meningitis | S                           | Korhonen <i>et al.</i> , 1984   |
| <i>H. influenzae</i> type B         | Anton                       | Van alphen <i>et al.</i> , 1986 |
| <i>Plasmodium knowlesi</i>          | Duffy                       | Miller <i>et al.</i> , 1975     |

was reported to be the receptor for fimbrial adhesins expressed on strains of *E. coli* often isolated from sufferers of pyelonephritis [Kallenius *et al.*, 1980].

If ABH or Le<sup>b</sup> antigens act as receptors for microorganisms, these antigens in the body fluids of secretors might bind to adhesins on the surface of microorganisms in a lectin-like interaction and block their attachment to the cells. This hypothesis was supported by the inhibition of attachment of *Strep. salivarius* to epithelial cells by salivary glycoproteins with blood group activity [Williams and Gibbons, 1975]. Also binding of candida to epithelial cells was inhibited by heat treated saliva from secretors but not saliva from non-secretors [Thom *et al.*, 1989].

The Le<sup>a</sup> antigen found predominantly on the cells of non-secretors might be a receptor for lectin-like adhesins of some microorganisms. The binding of uropathogenic strains of *E. coli* to target cells from non-secretors was greater compared with binding to secretor cells [Lomberg *et al.*, 1986]. Binding of *Candida* blastospores to non-secretor cells was inhibited by pretreating the cells with polyclonal anti-Le<sup>a</sup> but not anti-Le<sup>b</sup> [May *et al.*, 1989]. A study by Tosh and Douglas [1992] has identified an adhesin on *C. albicans* that binds to fucose containing blood group antigens. The fucose binding protein was isolated by an affinity adsorption technique and the purified protein inhibited yeast attachment to BEC efficiently.

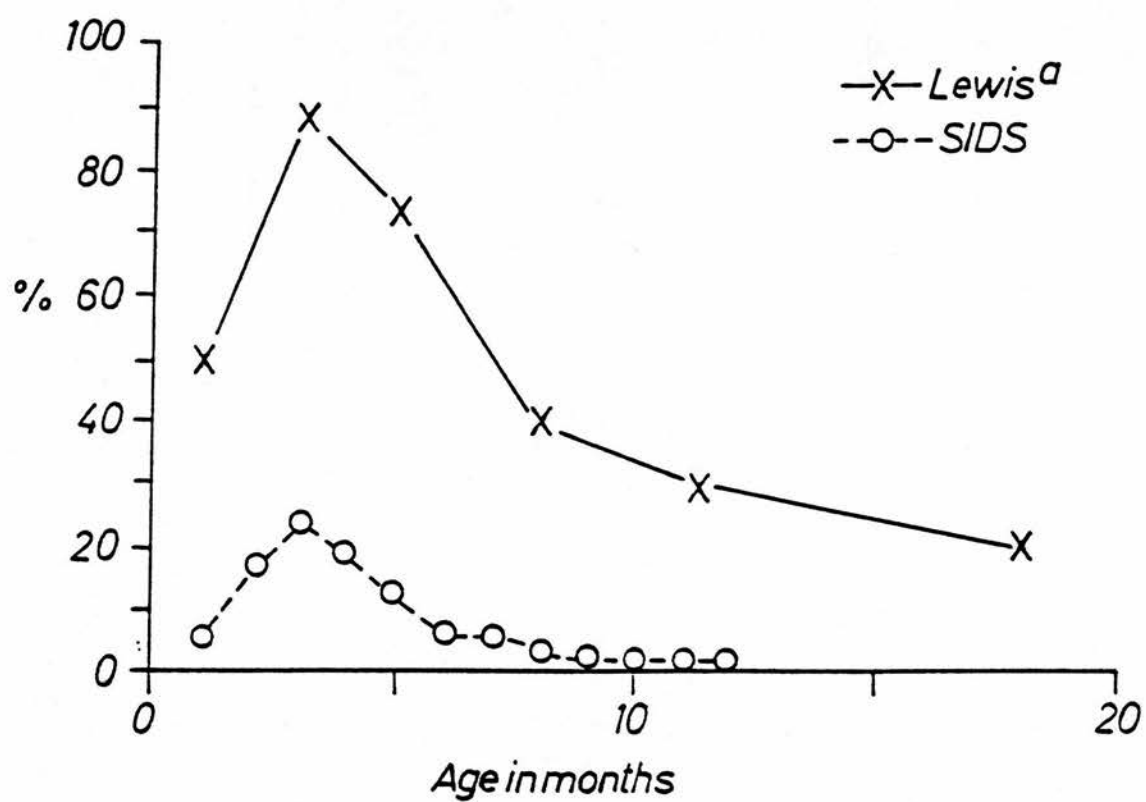
## **1.7 Aims of the study and hypothesis to be tested**

The most consistent feature of SIDS is the age distribution [Carpenter and Gardner, 1982]. The peak of SIDS is at 2-3 months of age then rapidly falls and the phenomenon is rare after the first year of life. This suggests that SIDS might be associated with a critical phase of development during which babies are at high risk [Milliner, 1987]. One of the most remarkable characteristics of the Lewis blood group system is the development and alteration of the phenotypes in infancy and childhood

[Jordal, 1956]. There are differences in maturation or efficiency of the products of the secretor gene and the Lewis gene during this period in the infants' development. Le<sup>a</sup> develops during the first postnatal weeks, its frequency of detection on red cells is greatest 2-3 months after birth. After that age, the frequency decreases, reaching the level found in adults from about the age of 2 years. Le<sup>b</sup> is more uncommon in children than in adults [Jordal, 1956]. The peak incidence for detection of Le<sup>a</sup> on erythrocytes of infants is coincident with a high incidence of SIDS (Figure 1.3) and in a study by [Aniansson *et al.*, 1992] nasopharyngeal colonization by *S. aureus* during the first three months of life was higher than the end of the first year.

Based on epidemiological findings and laboratory investigations, the aims of this thesis were to test the following hypothesis:

1. Le<sup>a</sup> antigen expressed on infants' cells is one of the receptors for toxigenic strains of *S. aureus* and *B. pertussis* suggested to be involved in SIDS.
2. RSV infection enhances binding of *S. aureus* and *B. pertussis* to epithelial cells.



**Figure 1.3 Analysis by age of expression of Le<sup>a</sup> antigen and prevalence of SIDS.**



## **Chapter 2**

### **General Materials and Methods**

All chemicals were analytical grade obtained mostly from BDH Chemicals Ltd.UK or Sigma UK.

#### **2.1 Buffers and solutions used for enzyme immunosorbent assays (ELISA)**

##### **2.1.1 Coating buffer (carbonate-bicarbonate buffer)**

Coating buffer consisted of sodium carbonate (15 mM), sodium bicarbonate (35 mM) and sodium azide (3 mM) (pH 9.6).

##### **2.1.2 Phosphate buffered saline (PBS)**

PBS contained sodium chloride (15 mM), sodium orthophosphate (3.7 mM) and disodium hydrogen phosphate (9.6 mM) (pH 7.2).

##### **2.1.3 Washing buffer**

Washing buffer consisted of (0.1 % w/v) bovine serum albumin (BSA) and (0.05 % v/v) Tween-20 in PBS (pH 7.2).

##### **2.1.4 Blocking buffer**

Blocking buffer was prepared by adding (1 % w/v) BSA to PBS.



### **2.1.5 Phosphate citrate buffer**

Phosphate citrate buffer contained sodium hydrogen phosphate (0.1 M) and citric acid (0.1 M) (pH 5).

### **2.1.6 Substrate solution**

The substrate was prepared by adding 40 mg of O-phenylenediamine to 100 ml of 0.1 M phosphate citrate buffer. It was activated immediately before use with 40 µl of hydrogen peroxide (30 % v/v).

### **2.1.7 Stopping solution**

The stopping solution is made of 12.5% (v/v) H<sub>2</sub>SO<sub>4</sub>.

## **2.2 Buccal epithelial cells (BEC)**

BEC were obtained from healthy students and staff (age range 20-64) in the Department of Medical Microbiology, University of Edinburgh. BEC were collected by gently scraping the lining of the mouth with cotton swabs. The swabs were agitated in (10 ml) of PBS. The cells were washed twice with PBS in a Sorval RT 6000 centrifuge at 300 g for 10 min. The BEC were counted with a Neubauer haemocytometer and their concentration was adjusted to  $2 \times 10^5$  cells per millilitre.

## **2.3 Collection of saliva**

Saliva (20 ml) was collected from the same individuals whose BEC were used. The saliva was centrifuged at 1000 g for 15 min. The supernates were transferred to glass universal containers and boiled for 30 min, centrifuged again and stored at -20°C.

## **2.4 Determination of ABO blood group**

The ABO blood group of the individuals were determined from finger prick blood specimens by slide agglutination with monoclonal anti-A and anti-B antibodies from the Scottish National Blood Transfusion Service, (SNBTS).

## **2.5 Determination of secretor status**

Secretor status was determined from a saliva specimen of each donor by haemagglutination inhibition assays [Mollison, 1983]. Saliva (25 µl) prepared in 2.3 was added to 25 µl of 3 appropriate dilutions of anti-A or anti-B (SNBTS) and 2 dilutions of the *Ulex europaeus* lectin (Sigma) in V-well micro-titre trays. The saliva of a non-secretor and of a secretor were included as controls. The plates were incubated at room temperature (RT) for 30 min after which the appropriate blood cells O, A1 mix or B (25 µl) (SNBTS) diluted 1/5 in saline were added to the wells. They were incubated at RT for 2 h or overnight at 4°C and examined for agglutination.

## **2.6 Determination of Lewis phenotype of erythrocytes**

Saline (2 ml) was added to 20 µl of blood in a Falcon test tube and centrifuged at 300 g for 5 min. The supernatant was taken off and 40 µl of saline was added to the test tube. The cells (20 µl) were added to 20 µl of anti-Le<sup>a</sup> (SNBTS) and 20 µl to an equal amount of anti-Le<sup>b</sup> (SNBTS). The tubes were incubated at RT for 30 min and observed for agglutination.

## **2.7 Purification of monoclonal anti-Lewis<sup>a</sup> and anti-Le<sup>b</sup>**

Synsorb affinity adsorbent (Chembiochem Ltd., Edmonton, Canada) with the synthetic carbohydrate determinant of Le<sup>a</sup> or Le<sup>b</sup> covalently linked to the silica matrix was used to purify the anti-Le<sup>a</sup> and anti-Le<sup>b</sup> monoclonals. Hybridoma culture supernatant (20 ml) containing mouse anti-Lewis<sup>a</sup> antibody (LM 112/161) or anti-Le<sup>b</sup> (LM 112/81) antibodies (kindly provided by Dr. R. Fraser, Glasgow and West Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, UK) was mixed with 1 g of the corresponding Synsorb beads overnight at 4°C. The unbound antibodies were removed and the beads were washed twice with PBS by centrifugation at 50 g for 5 min. The bound material was eluted by adding 5 ml of 2% (v/v) ammonia (BDH, 35%) in saline (pH 11) to the beads for 15 min at RT and centrifuged at 50 g for 5 min. The supernatant was dialysed against PBS overnight and stored at - 20°C. The washed beads were stored in ethanol (70 % v/v) at 4°C.

## **2.8 Bacteria**

*S. aureus* strains used in this study, the toxins they produce and their site of isolation are listed in Table 2.1. National Culture Type Collection (NCTC) strains NCTC 10652, NCTC 10654, NCTC 10655, NCTC 10656, NCTC 10657, NCTC 11965 and NCTC 8532 were obtained from Dr. A. Wieneke, Central Public Health Laboratory, Colindale. Strains 40654 and 41206 were kindly provided by Dr. J Medcraft, Public Health Laboratory Service, Department of Microbiology, Reading, Berkshire.

**Table 2.1 Toxins produced and source of *S. aureus***

| <b><u>Strain number</u></b> | <b><u>Toxin produced</u></b> | <b><u>Source</u></b>    |
|-----------------------------|------------------------------|-------------------------|
| NCTC 8532                   | None                         | Not known               |
| NCTC 10652                  | A                            | Ham                     |
| NCTC 10654                  | B                            | Faeces                  |
| NCTC 10655                  | C                            | Leg abscess             |
| NCTC 10656                  | D                            | Turkey salad            |
| NCTC 10657                  | A and B                      | Not known               |
| NCTC 11965                  | A and TSST-1                 | Vaginal swab            |
| 40654                       | A                            | Nose                    |
| 41206                       | B                            | Post-mortem lung tissue |

### **2.8.1 Culture media and storage of bacteria**

The bacteria were grown on nutrient agar plates or, for some experiments, on blood agar plates to examine the effect of medium on binding of bacteria to epithelial cells. They were incubated in a humidified aerobic atmosphere with 5% CO<sub>2</sub> for 24h at 37°C. For storage, colonies were emulsified in Microbank beads and kept at -20°C. A fresh bead was used to inoculate plates for each set of experiments.

### **2.8.2 Preparation of bacteria**

*S. aureus* strains were subcultured as described (2.8.1) and colonies were emulsified in 10 ml of PBS and centrifuged at 1000 g for 20 min. The bacterial pellet was resuspended in 2 ml of PBS.

### **2.8.3 Total bacterial count**

After measuring the optical density (OD) of two fold dilutions of the washed suspension for each strain at 540 nm with a spectrometer (Pye Unicam), the total number of bacteria was determined in a counting chamber (Thoma) by light microscopy. A graph of the OD versus total count was prepared for each strain.

## **2.9 Buffers used for determination of bacterial binding to buccal epithelial cells**

### **2.9.1 Fluorescein isothiocyanate (FITC) buffer**

The buffer contained sodium carbonate (0.05 M) and sodium chloride (0.1 M) (pH 9.2). FITC (0.4 mg/ml)(Sigma) was dissolved in the buffer immediately before use.

## **2.9.2 Buffered paraformaldehyde**

Buffered paraformaldehyde (1% v/v) was prepared by adding paraformaldehyde (BDH, 97%) to sodium cacodylate (1% w/v) and sodium chloride (0.75% w/v).

## **2.10 Bradford reagent for protein estimation**

Bradford reagent was prepared from Commasie Blue G250 (0.01% w/v) (Sigma), ethanol (4.7% w/v) and phosphoric acid (8.5% w/v) in distilled water.

## **2.11 Monoclonal and polyclonal antibodies**

Monoclonal and polyclonal antibodies, the animal from which they were obtained, isotype and source are listed in Tables 2.2 and 2.3.

**Table 2.2 Monoclonal antibodies**

| <u>Monoclonal antibody</u>                             | <u>Host</u> | <u>Isotype</u> | <u>Source</u>                      |
|--|-------------|----------------|------------------------------------|
| Anti-A   | mouse       | IgG            | SNBTS                              |
| Anti-B   | mouse       | IgG            | SNBTS                              |
| Anti-Le <sup>a</sup> (Red cell typing)                 | mouse       | IgM            | SNBTS                              |
| Anti-Le <sup>a</sup> (Inhibition of bacterial binding) | mouse       | IgM            | SAPU                               |
| Anti-Le <sup>b</sup> (Red cell typing)                 | mouse       | IgM            | SNBTS                              |
| Anti-Le <sup>b</sup> (Inhibition of bacterial binding) | mouse       | IgM            | SAPU                               |
| Anti-precursor type 1                                  | mouse       | IgM            | Russel Fine Chemicals,<br>Chester. |
| Anti-CD15 (anti-Le <sup>x</sup> )                      | mouse       | IgM            | Serotec                            |

**Table 2.3 Polyclonal antibodies**

| <u>Polyclonal antibody</u>        | <u>Host</u> | <u>Isotype</u> | <u>Source</u> |
|-----------------------------------|-------------|----------------|---------------|
| Anti-Le <sup>a</sup>              | goat        | IgG            | Behring       |
| Anti-Le <sup>b</sup>              | goat        | IgG            | Behring       |
| *HRP-labelled anti sheep/Goat     | donkey      | IgG            | SAPU          |
| *HRP-labelled anti-rabbit         | donkey      | IgG            | SAPU          |
| FITC anti-mouse                   | goat        | IgM            | Sigma         |
| Anti-staphylococcal enterotoxin-B | rabbit      | IgG            | Sigma         |

\* HRP = horse radish peroxidase.



## Chapter 3

# Binding of *Staphylococcus aureus* to BEC of secretors and non-secretors

### 3.1 Introduction

*S. aureus* is part of the normal flora inhabiting the nasopharynx and the oropharynx of a significant proportion of healthy humans [Rosebury, 1962]. Adherence of *S. aureus* to epithelial cells is achieved partly by non-specific mechanisms; the electric charge and hydrophobicity of bacterial cell walls vary during growth of *S. aureus* and influence their adherence to epithelial cells [Beck *et al.*, 1988]. In addition, there are specific "lock and key" bonds between complementary molecules on cell surfaces of the host and the bacteria. *S. aureus* cell wall components, teichoic acids and protein A, have been implicated in adherence [Cole and Silverberg, 1986; Wyatt, 1990]. Teichoic acids bind specifically to nasal epithelial receptor sites for *S. aureus* [Aly *et al.*, 1980], and lipoteichoic acid mediates adherence of *S. aureus* to BEC [Carruthers and Kabat, 1983]. The epithelial cell receptors for *S. aureus* are poorly understood; however, results indicate that the preferential attachment of *S. aureus* to endothelial cells is mediated by fibrinogen absorbed from plasma on to the endothelial surface [Cheung *et al.*, 1991]. Sites at which *S. aureus* typically initiates infection are rich in fibronectin, for example blood clots and sub-endothelium [Proctor, 1987].

Epidemiological studies have found that individuals who are non-secretors of the ABO blood group antigens are over-represented among patients with some bacterial diseases and among carriers of some potentially pathogenic bacteria. One hypothesis suggested to explain these epidemiological associations is that the Le<sup>a</sup> blood group antigen expressed predominantly on the cells of non-secretors is one of the receptors for some

strains of bacteria. During the age range (2-4 months) in which the peak incidence of SIDS occurs, 80-90% of infants express Le<sup>a</sup> (Figure 1.3). The objective of this study was to develop a reliable method for assessment of bacterial binding and to test the hypothesis that toxigenic strains of *S. aureus* isolated from SIDS infants bind in greater numbers to cells from non-secretor donors which express more Le<sup>a</sup> than cells from secretor donors.

## **3.2 Materials and methods**

### **3.2.1 Epithelial cells**

Buccal epithelial cells were obtained from pairs of healthy secretor and non-secretor donors matched as closely as possible for ABO blood groups, age and sex. The cells were washed and adjusted to  $2 \times 10^5$  cell / ml as described in (2.2).

### **3.2.2 Labelling of *S. aureus* with FITC or rhodamine**

Bacteria were labelled with FITC by a modification of the method of Wright and Jong [1986]. A heavy suspension of bacteria was prepared and washed twice by centrifugation at 1000 g for 20 min. The bacterial pellet was resuspended in 4 ml of freshly prepared FITC (2.9.1). The mixture was incubated at 37°C for 20 min and washed twice with PBS. The pellet was resuspended in PBS and filtered through a Millipore membrane filter (5 µm pore diameter) and the concentration adjusted by OD at 540 nm (2.8.3).

Rhodamine 123 (Sigma, UK.) was also used to label the bacteria. Rhodamine (1 mg/ml PBS) (2 ml) was added to a pellet of *S. aureus* and incubated for 20 min at RT. The

bacteria were washed twice in PBS, filtered and the concentration adjusted as described for the FITC labelling procedure.

### **3.2.3 Methods for detection of the attachment of *S. aureus* to BEC**

#### **3.2.3.1 Light Microscopy**

A binding assay described by [Bagg *et al.*, 1982] was used. Samples of a suspension of *S. aureus* strain NCTC 10655 ( $2 \times 10^8$ /ml) were mixed with equal volumes of a buccal cell suspension ( $2 \times 10^5$ /ml) in glass universal tubes. They were rotated at 30 rpm at 37°C for 45 min; each mixture was filtered through a Millipore membrane filter (5 µm pore size) to remove the unbound bacteria. Each filter was washed with 50 ml of PBS and inverted onto a drop of PBS on a microscope slide. The filters were removed after 2 min. The slides were air-dried, fixed in methanol for 5 min and finally stained by Gram's stain. Bacterial counting was performed at a magnification of 400 under oil immersion.

#### **3.2.3.2 Detection of bacterial binding to BEC by Confocal Scanning Optical Microscopy (CSOM)**

*S. aureus* strain NCTC 10655 was used in this study. BEC (200 µl) were mixed with 200 µl of the FITC-labelled bacteria at different ratios of bacteria per cell. The mixtures were incubated at 37°C for 30 min with gentle shaking at 60 rpm in an orbital incubator (Gallenkamp). The cells were washed twice with PBS by centrifugation at 300 g for 10 min to remove unattached bacteria and resuspended in 200 µl of PBS and 200 µl of 1% (v/v) buffered paraformaldehyde then stored in the dark at 4°C until analysed. In-order to remove the salt crystals seen under the microscope, the cells were washed once more with distilled water before the analysis.

Dry and wet preparations were examined by CSOM. To select suitable cells, a field was first scanned at low magnification with transmitted white light; ultraviolet (UV) light was then used to identify cells which had bound FITC-labelled bacteria. Images of individual cells obtained at a magnification of 1600 by reflected white light were saved on the hard disc. The fluorescence mode was again selected (488 nm argon ion laser) and images obtained in this mode were saved. Coloured overlays of the two sequences of images were then made and photographed. Finally a series of 45 optical sections of single cells, "cut" at 1  $\mu$ m intervals were downloaded to a silicon graphics workstation allowing 3-dimensional (3D) representations of the cell to be made by the programme, Voxel View. Each cell could be surveyed from any direction; the surface to which bacteria were bound could then be identified with precision.

### **3.2.3.3 ELISA to detect bound bacteria with antibody**

A modified method of Morrin and Reen (1993) was used. Samples of washed BEC in PBS were added to a microtitre plate. The monolayer of buccal epithelial cells ( $2 \times 10^5$ ) was dried on to the plates overnight at 37°C and fixed by treatment with glutaraldehyde (Sigma) 0.25 % for 10 min. The wells were washed 3 times with PBS. Suspensions of *S. aureus* strain NCTC 10657 grown on nutrient agar plates for 24 h were added at ratios of 640 and 1280 bacteria per BEC and incubated for 1 h at 37°C. After washing 3 times with PBS, the cells were fixed with glutaraldehyde and blocked by BSA. The BSA was removed and bound *S. aureus* were detected with rabbit anti-staphylococcal enterotoxin B (Sigma, S 9008) diluted 1/500 in PBS. The antibody was allowed to react for 1 h at RT. After washing, 100  $\mu$ l of HRP-labelled anti-rabbit IgG (SAPU) diluted 1/250 in PBS were added to the wells and incubated for 2 h at RT. The wells were washed with PBS and 50  $\mu$ l of the substrate solution (2.1.6) were added. The reaction was stopped after 20 min by adding 50  $\mu$ l of 12.5% ( v/v )  $H_2SO_4$ . The optical density at 450 nm was measured with a Dynatech plate reader.

For the controls PBS was added in place of the anti-staphylococcal enterotoxin B or PBS was added in place of bacteria.

### **3.2.3.4 Analysis of binding assays by flow cytometry**

The binding studies were carried out with FITC-labelled or rhodamine 123-labelled staphylococci (3.2.2).

BEC (200  $\mu$ l) were mixed with 200  $\mu$ l of the labelled bacteria at the desired ratios of bacteria per cell. The mixtures were incubated at 37°C for 30 min with gentle shaking in an orbital incubator (Gallenkamp). The cells were washed twice with PBS by centrifugation at 300 g for 10 min to remove unattached bacteria. The samples were resuspended in 300  $\mu$ l of buffered paraformaldehyde (Sigma) and EPICS C flow cytometer (Coulter Electronics, Ltd) was used in these studies. BEC were selected from a display of forward angle light scatter which is proportional to the size of the particle versus 90° light scatter which is proportional to the granularity of the particle by means of a bit map. The bit map included the main population of the cells and excluded debris and clumps from further analysis. At least 2000 BEC were analysed in each sample. The percentage of fluorescence in a FITC-labelled sample was assessed with reference to a cursor placed in a channel where less than 2% of the cells in an unlabelled sample were positive. The percentage of cells with fluorescence greater than the background was recorded on a one-parameter histogram, measuring fluorescence on a logarithmic scale. The mean fluorescence channel values for the positive cells were obtained from a one-parameter histogram measuring, fluorescence on a linear scale.

The results were analysed by immunoanalysis (Coulter), a computer programme that subtracts the values of the control population from the test population at each channel of the two histograms. The binding index (BI) for each sample was calculated by



multiplying the percentage of fluorescent cells by the mean fluorescence channel value. This index of total fluorescence in a sample gives an indication of the FITC-labelled staphylococci bound to the BEC.

### **3.2.4 Statistical methods**

All analyses were carried out on the logarithms of the binding indices which conformed more closely to a normal distribution than the raw values. Differences between groups were tested by paired t-test as appropriate and confidence limits for the mean values in one group were expressed as a percentage of those in the other by taking antilogarithms.

## **3.3 Results**

### **3.3.1 Correlation of total bacterial count with optical density**

The correlation between the readings of OD with the total bacterial count (2.8.3) for each strain of *S. aureus* tested is shown in Figure 3.1.

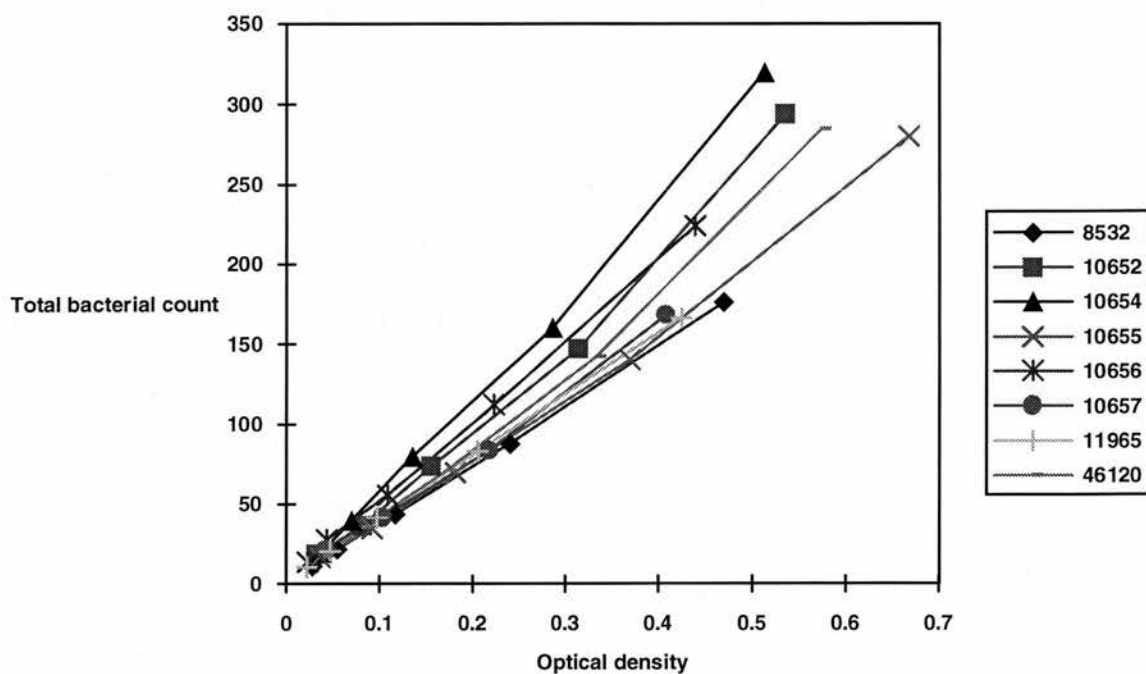


Figure 3.1 Total bacterial count ( $\times 10^{-7}$ ) determined by microscopy versus optical density of *S. aureus* strains tested.



### **3.3.2 Light microscopy**

Binding experiments with *S. aureus* at 3 different ratios (100, 400 and 1200) of bacteria per cell were assessed by light microscopy. Bacteria were not evenly distributed over the epithelial cells. The untreated epithelial cells used as control revealed many structures, the shapes and sizes of which could be mistaken for bacteria. Forty cells were counted on each slide. The data from duplicate slides were highly variable and there did not appear to be a correlation between the ratios of bacteria used and the number of bacteria observed attached to the cells (Table 3.1).

### **3.3.3 Confocal Scanning Optical Microscopy**

CSOM has the ability to superimpose two images of the same object one with and one without the laser mode. The number of bacteria bound to cells was related to the concentrations of bacteria used, 640:1 and 2560:1 (Figure 3.2 and Figure 3.3). Variations in the number of bacteria bound to cells were also evident for each concentration. A 3 dimensional view of the epithelial cell clearly demonstrated that the bacteria were attaching to its surface (Figure 3.4).



**Table 3.1. The mean of number of bacteria per cell counted by light microscopy.**

|                      | <u>Bacteria / Cell</u> | <u>Mean bacteria / cell</u> |
|----------------------|------------------------|-----------------------------|
| Unlabelled           | 100                    | 48                          |
| FITC-Labelled        | 100                    | 50                          |
| Unlabelled           | 400                    | 67                          |
| FITC-Labelled        | 400                    | 58                          |
| Unlabelled           | 1200                   | TNTC*                       |
| FITC-Labelled        | 1200                   | TNTC*                       |
| BEC without bacteria | 0                      | 24                          |

\* Too numerous to count

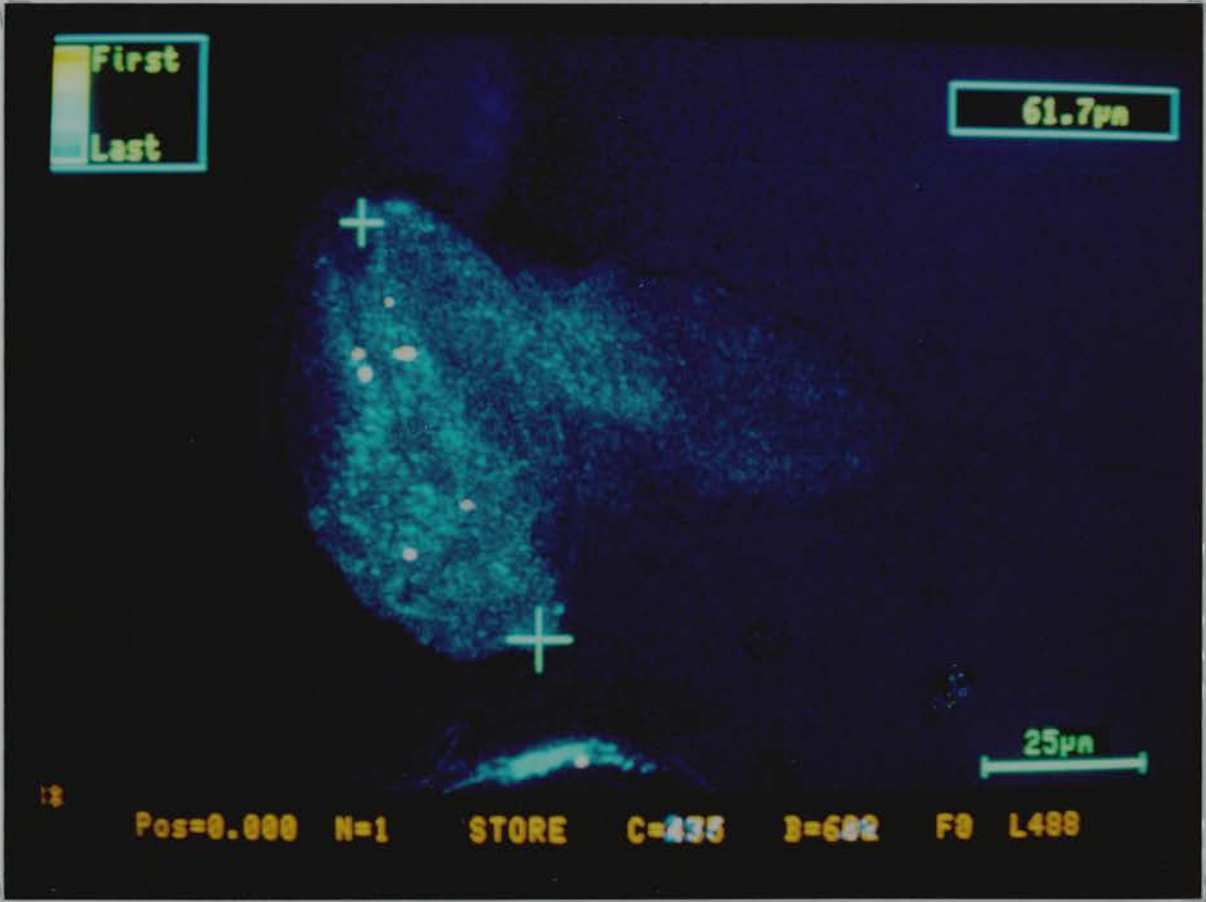


Figure 3.2 Detection of FITC-labelled *S. aureus* NCTC 10655 on BEC (640 B/C).

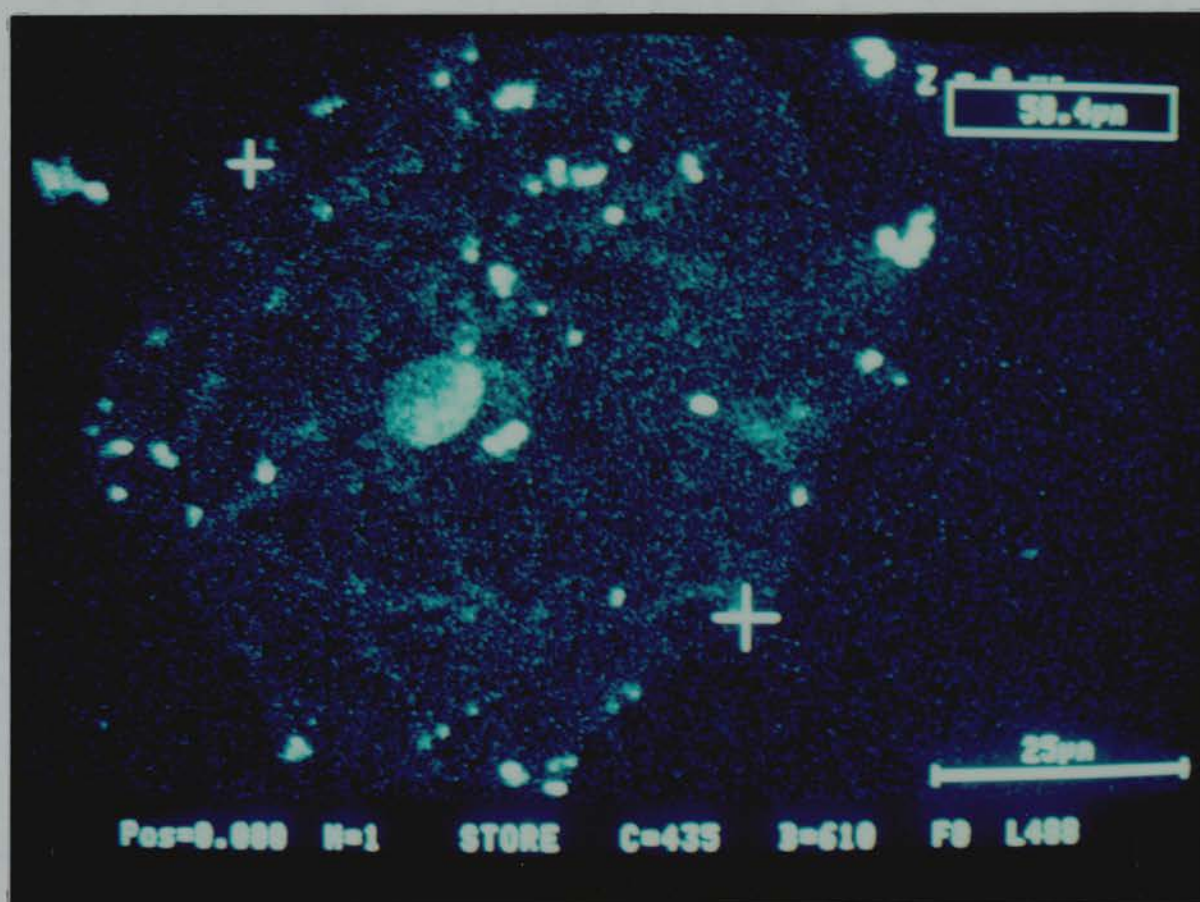


Figure 3.3 Detection of FITC-labelled *S. aureus* NCTC 10655 on BEC (2560 B/C).

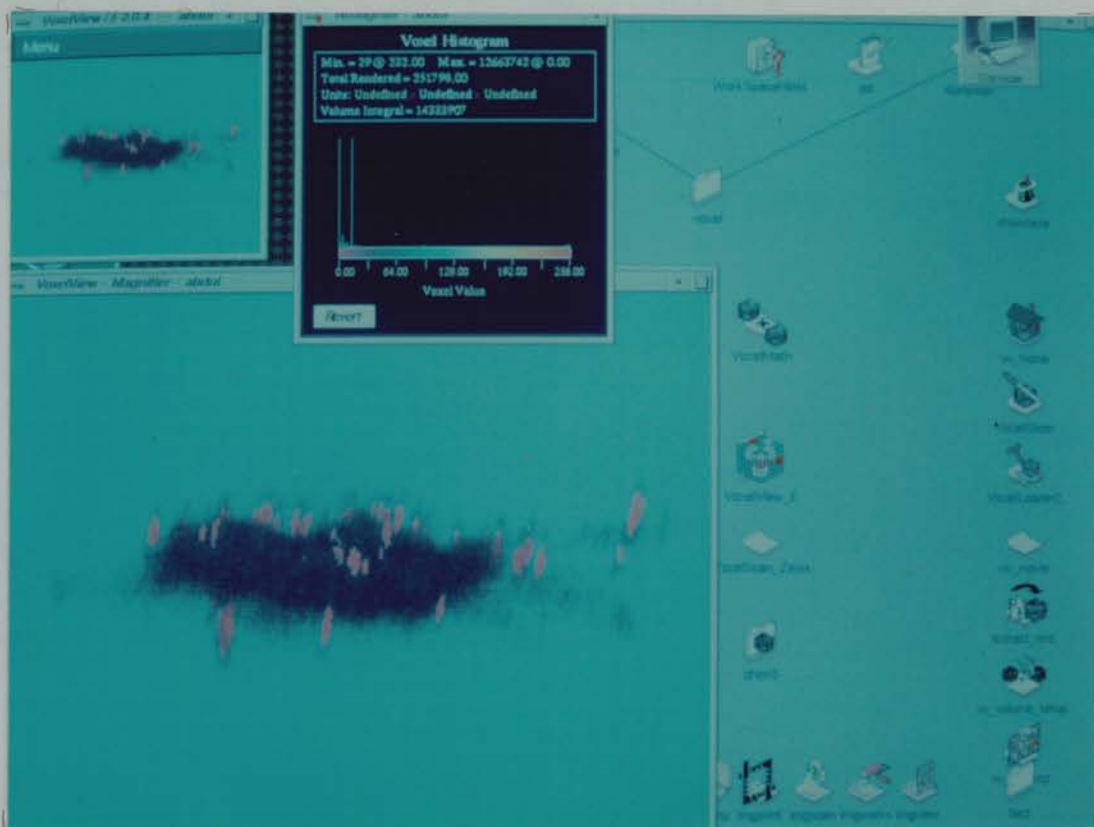


Figure 3.4 Three dimensional image of FITC-labelled bacteria on the surface of BEC.

### 3.3.4 ELISA to detect bound bacteria

*S. aureus* strain 10657 produces enterotoxins A and B. Rabbit anti-staphylococcal enterotoxin B was used to detect the bound bacteria. Both FITC-labelled and unlabelled bacteria were used. Two controls were included. One without bacteria and one without the anti-toxin B. The mean of 3 values for the labelled and unlabelled bacteria were similar for the 2 concentrations of the bacteria used, but there was significant binding of the antibody to cells to which no bacteria were added. This indicated that anti-toxin binds to structures on the BEC or to commensal bacteria (Table 3.2).

If the background for the cells with no bacteria (0.400) was subtracted from the values for cells to which bacteria were added, the results showed a dose response pattern with greater OD values for 1280 bacteria / cell (0.220, 0.217) compared with 640 bacteria / cell (0.120, 0.136).

### 3.3.5 Analysis of bacterial binding by flow cytometry

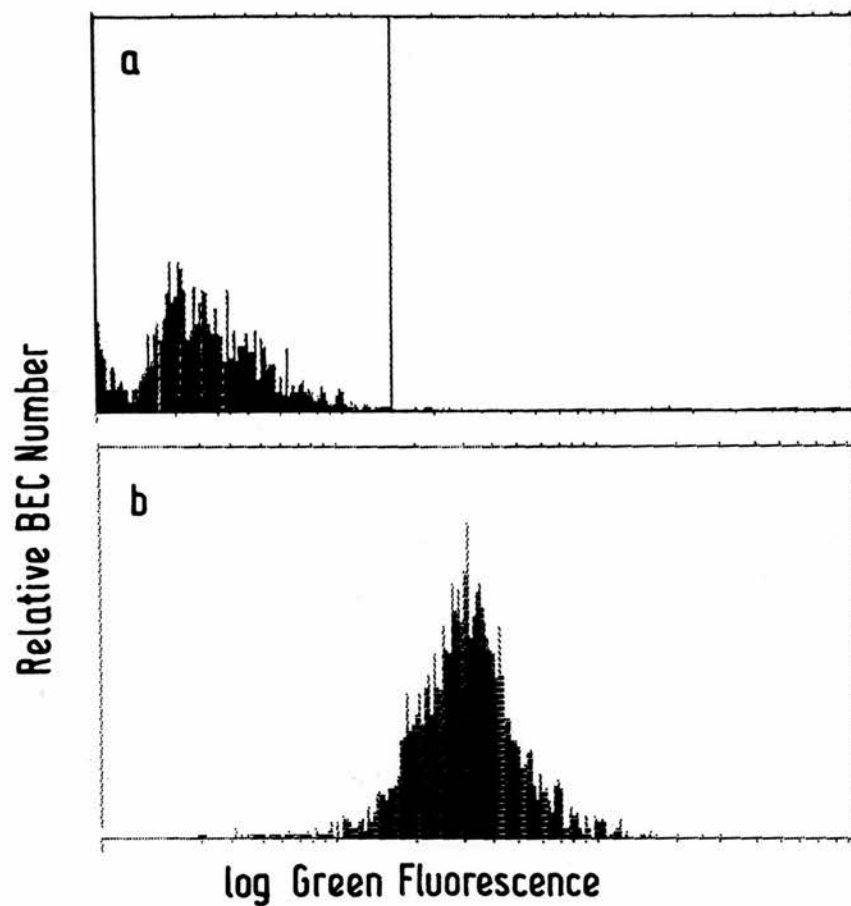
#### 3.3.5.1 Labelling of *S. aureus*

The peak in Figure 3.5b is an example of fluorescence observed for cells to which FITC-labelled bacteria are bound compared to control cells with no labelled bacteria Figure 3.5a. The binding indices for *S. aureus* labelled with FITC was much higher than those for cells with rhodamine 123. The results of one experiment in which both fluorochromes were used separately to label *S. aureus* (NCTC 10655) and added to BEC at ratios of 75, 150, 300 and 600 bacteria / cell are shown in Figure 3.6.

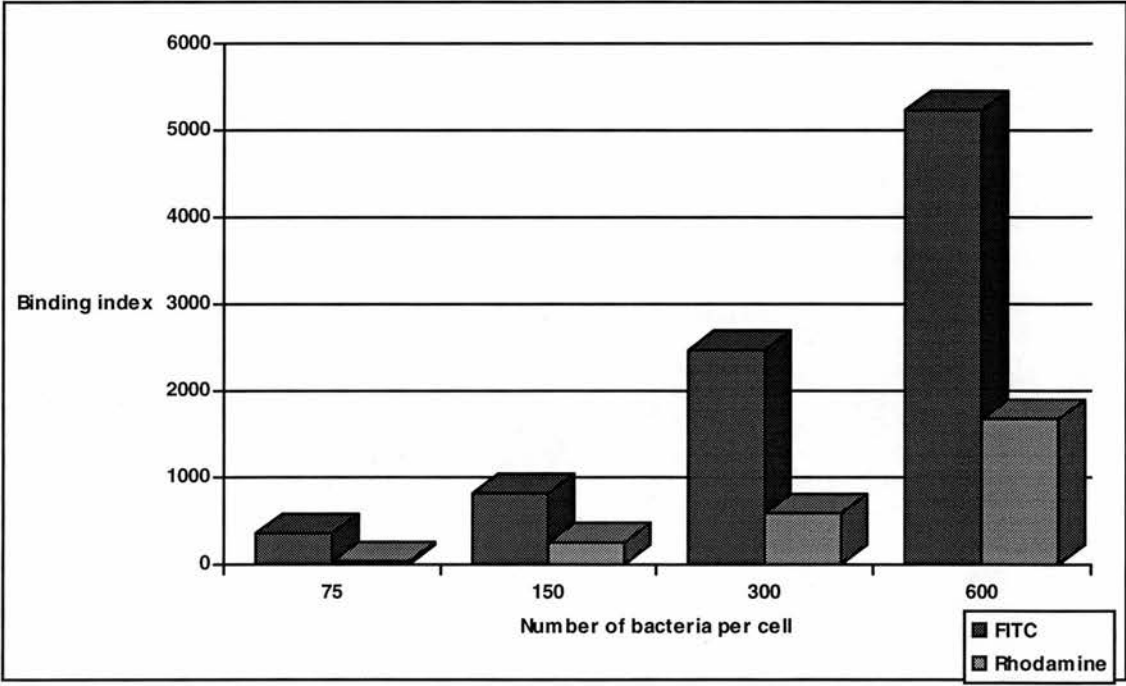
**Table 3.2 Mean OD values for ELISA with anti-enterotoxin B to detect bacterial binding.**

| <u>Bacteria / cell</u> | <u>Un-labelled bacteria (OD)</u> | <u>FITC-labelled bacteria (OD)</u> |
|------------------------|----------------------------------|------------------------------------|
| 0                      | 0.400                            | 0.400                              |
| 640                    | 0.520                            | 0.536                              |
| 1280                   | 0.620                            | 0.617                              |





**Figure 3.5** Flow cytometric analysis of bacterial binding to BEC using FITC conjugated bacteria (b) compared to cells with no bacteria (a).



**Fig 3.6 Binding indices for FITC or rhodamine labelled *S. aureus* bound to BEC.**

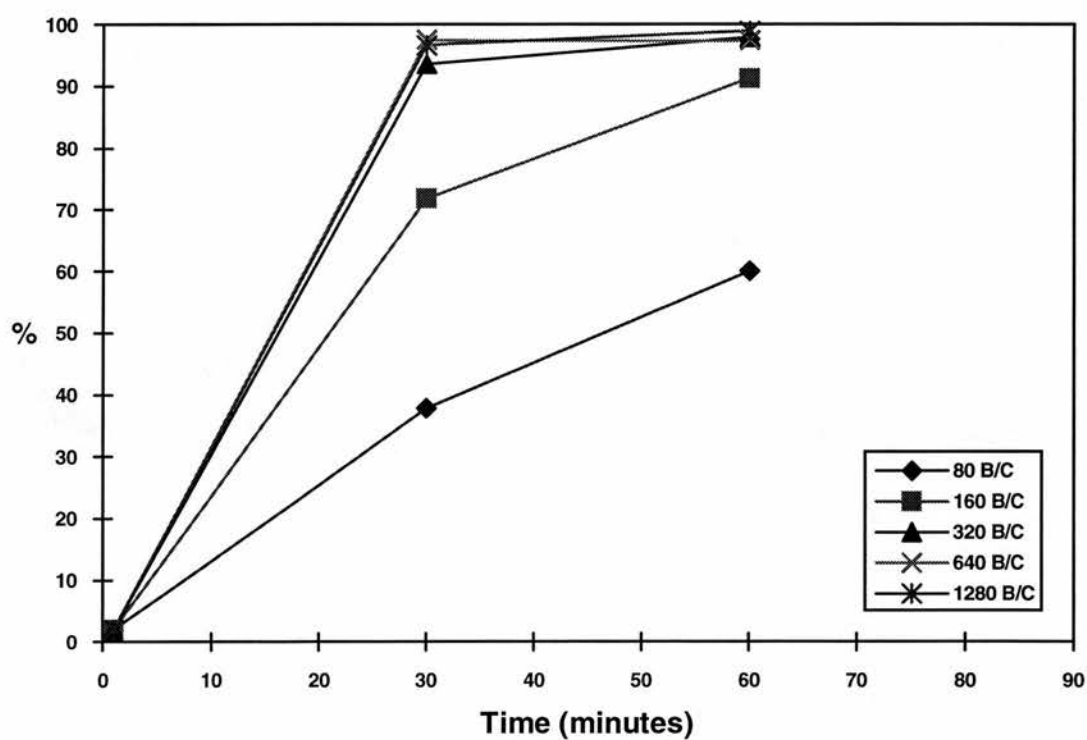


### **3.3.5.2 Optimal time for bacterial binding**

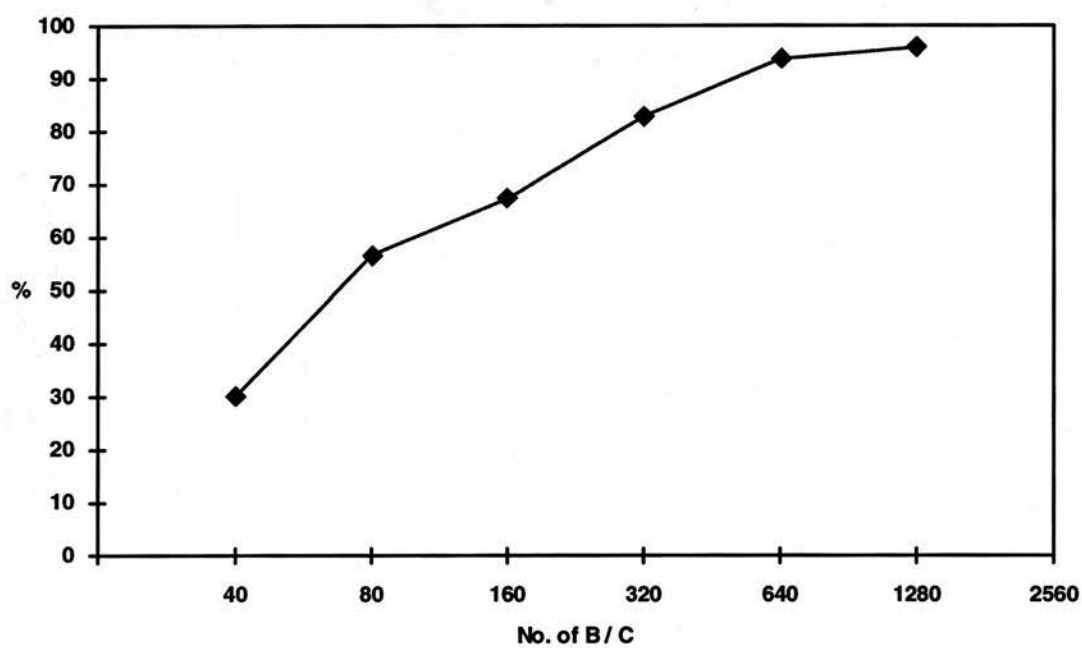
Different concentrations of FITC-labelled bacteria were incubated with BEC for different time periods and the attachment study was performed as described in 3.2.3.4. No fluorescence above background levels was observed when bacteria were added to BEC immediately prior to analysis. Attachment increased for the first 30 min then the increase was less rapid as is shown in Figure 3.7 As the attachment was adequate at 30 min, this was used as the standard incubation period for the binding assays.

### **3.3.5.3 Bacterial binding at different ratios of bacteria/cell**

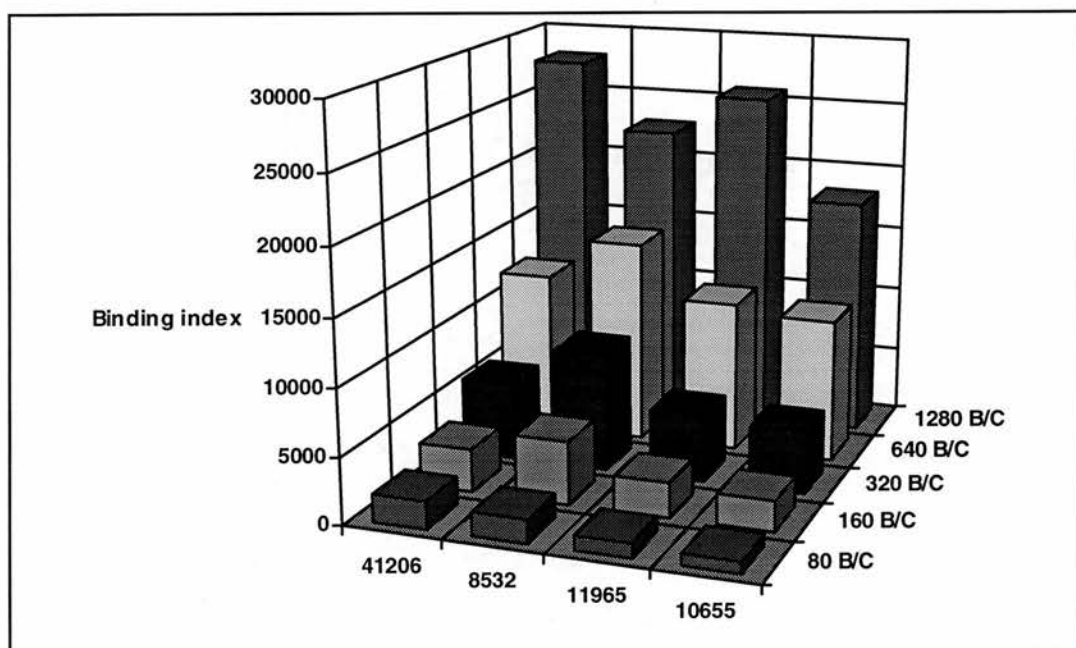
Attachment of *S. aureus* (Strain 10655) to BEC was examined with six different ratios of bacteria per cell (40, 80, 160, 320, 640, or 1280 : 1). Fig 3.8 illustrates the dose response effect of bacterial binding to cells from both a secretor and a non-secretor individual. Bacterial attachment was evident from 80 bacteria per cell and ratios of 80, 160, 320 and 640 bacteria per cell were used for further experiments. Fig 3.9 presents the binding of 4 strains of *S. aureus* to epithelial cells of the same donor. Similar patterns were observed with other strains.



**Figure 3.7** Time course of binding for *S. aureus* (NCTC 10655) to BEC (% is the percentage of cells being fluorescinated above background; B/C = bacteria/cell).



**Figure 3.8 Dose response of *S.aureus* (NCTC 10655) binding to BEC.**



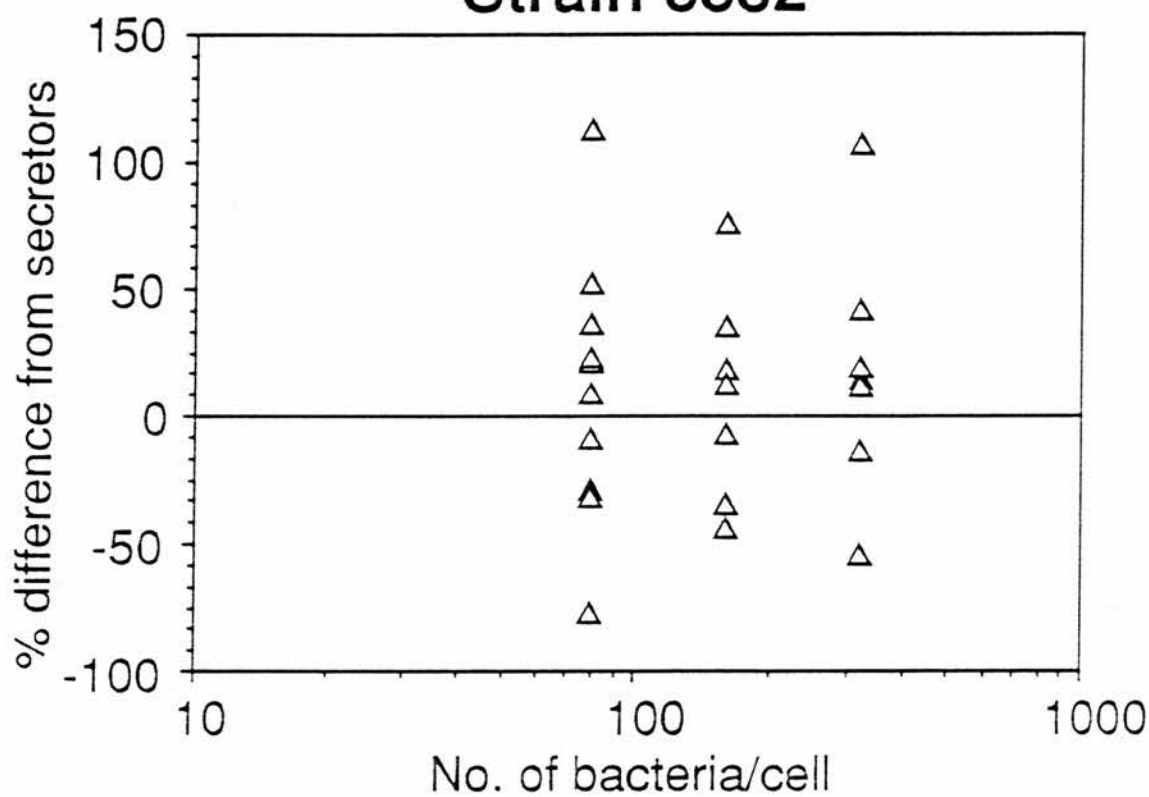
**Figure 3.9 Binding of four strains of *S. aureus* (41206, NCTC 8532, NCTC 11965 and NCTC 10655) to BEC of the same donor (B/C = bacteria / cell).**

### 3.3.6 Binding of *S. aureus* strains to BEC from secretors and non-secretors

Eight pairs of secretors / non-secretor donors were used for each experiment. In (Figures 3.10-3.18) the data are presented as a difference in the percentage of the binding (binding indices) between secretor and non-secretor cells, each point representing the results for one pair of donors. If there was no difference the point is on the line (0%). The points above the line represent those pairs in which the non-secretor cells bound more bacteria than cells from the secretor. The points below the line represent those pairs in which the secretor cells bound more than those from the non-secretor. There was no significant difference in binding to cells of non-secretors compared to cells of secretors observed for the non-toxigenic strain NCTC 8532 (Figure 3.10) or five of the toxin producing strains: NCTC 41206 (Figure 3.11); NCTC 40654 (Figure 3.12); NCTC 10652 (Figure 3.13); NCTC 10654 (Figure 3.14); or NCTC 10656 Figure (3.15). Three of the toxin producing strains, NCTC 10655, NCTC 10657 and NCTC 11965, showed higher binding to cells from non-secretors compared with binding to cells from secretors (Figures 3.16, 3.17 and 3.18). There was significantly higher binding of NCTC 10655 and NCTC 10657 to non-secretors' cells at all ratios of bacteria: cells tested; for strain NCTC 11965, there was significantly higher binding to cells of non-secretors only with the lowest ratio of bacteria.

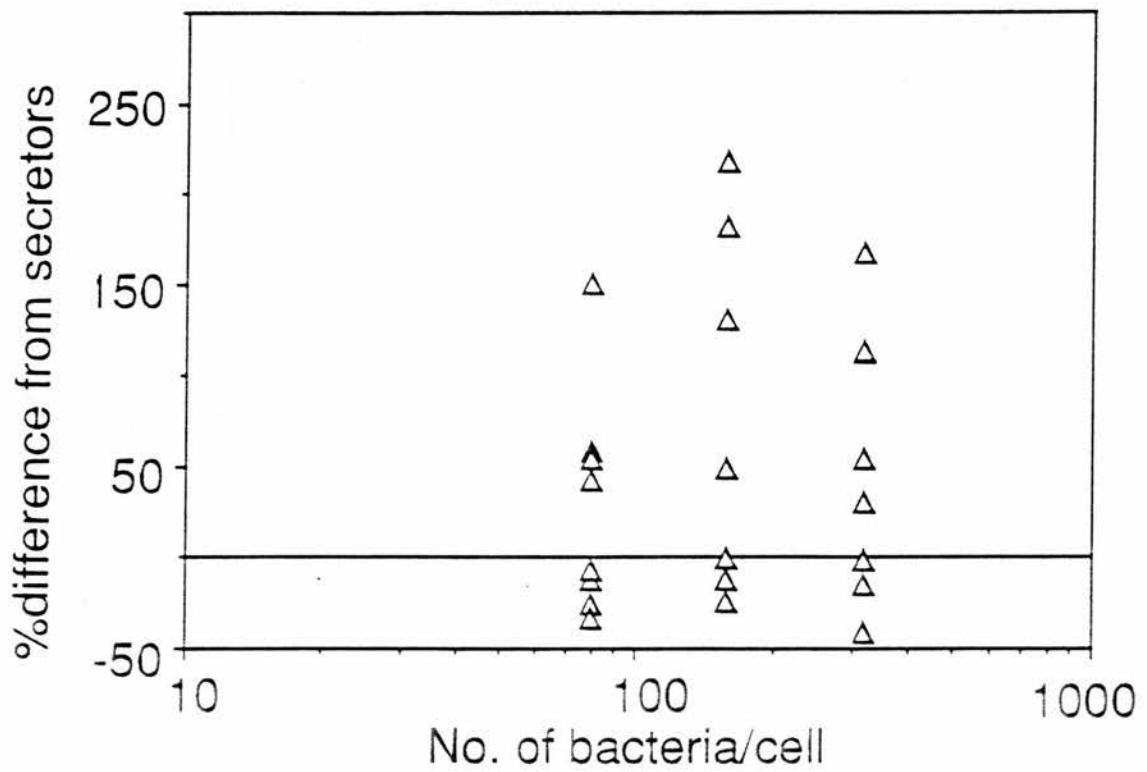
Table 3.3 summarises the results for the strains tested by the paired *t* test. The 95% confidence limits for binding of the bacteria to non-secretor cells are expressed as a percentage of binding to cells from matched secretors. The confidence intervals for which both values are above 100 indicated significantly higher binding of bacteria to cells from non-secretors than cells from secretors.

## Staphylococcus aureus Strain 8532



**Figure 3.10 Binding of *S. aureus* (NCTC 8532) to epithelial cells of secretors and non-secretors.**

## Staphylococcus aureus Strain 41206



**Figure 3.11** Binding of *S. aureus* (41206) to epithelial cells of secretors and non-secretors.

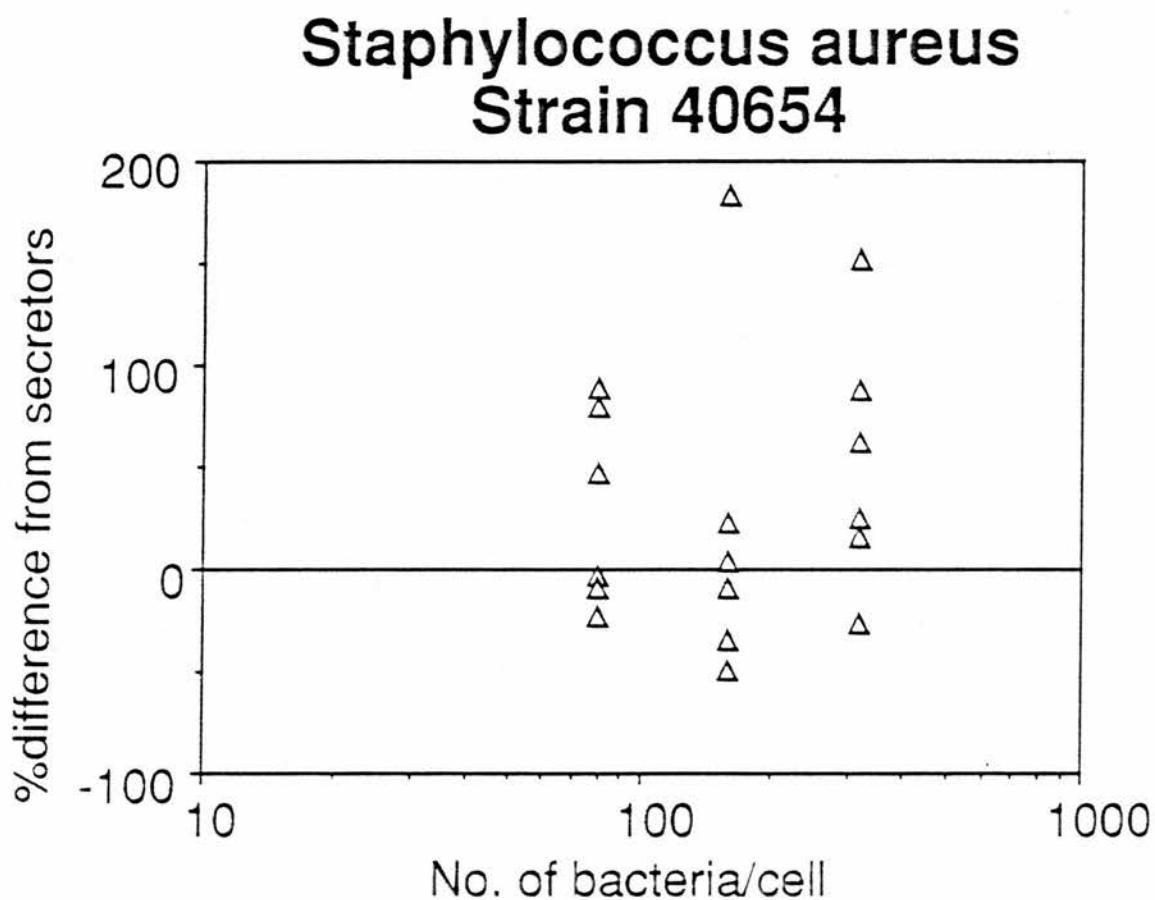
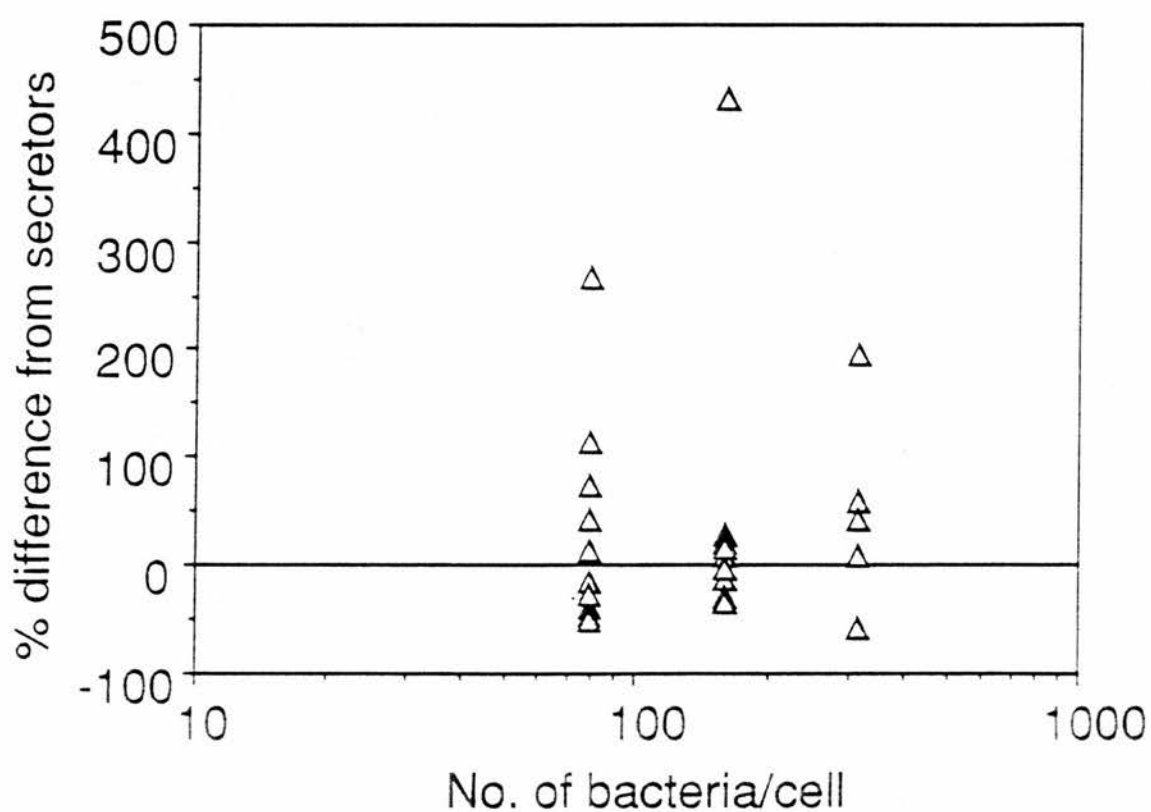


Figure 3.12 Binding of *S. aureus* (40654) to epithelial cells of secretors and non-secretors.

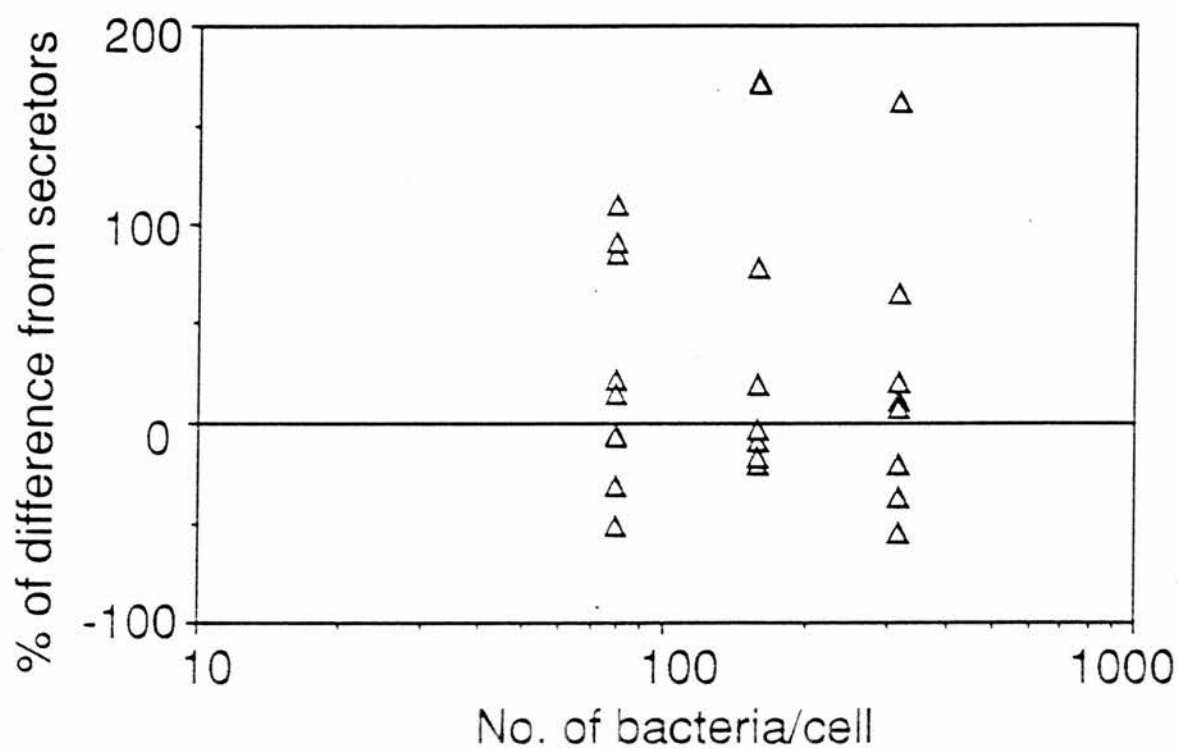


## Staphylococcus aureus Strain 10652



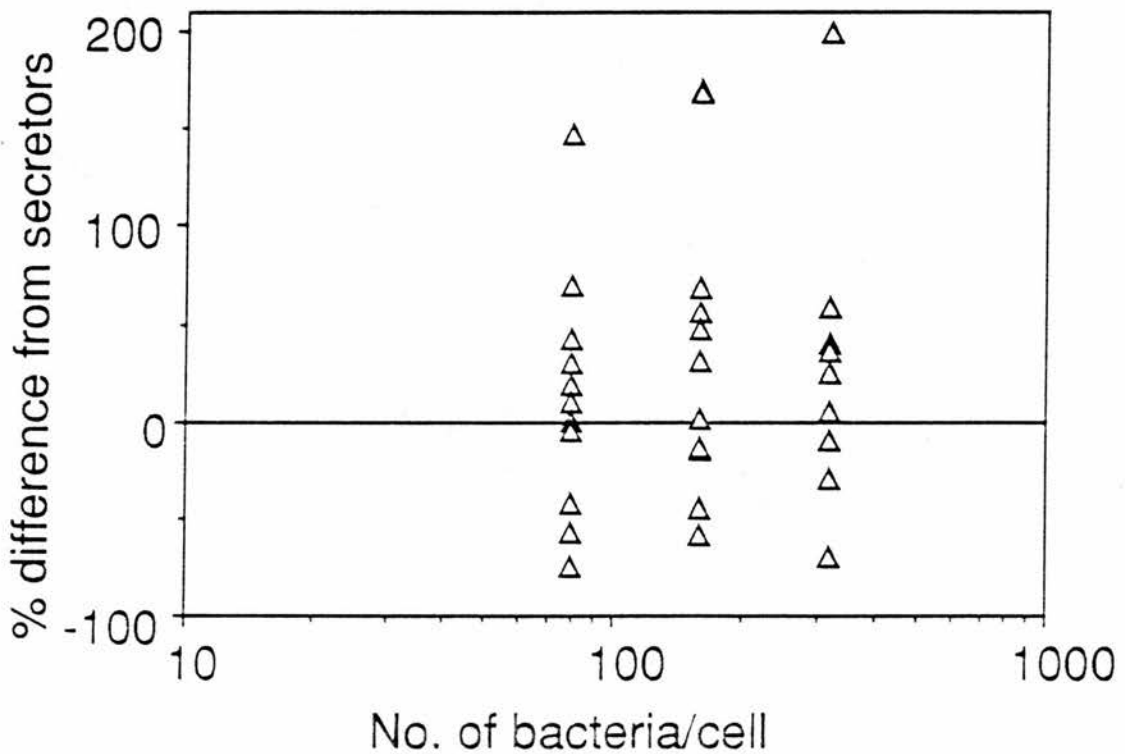
**Figure 3.13 Binding of *S. aureus* (NCTC 10652) to epithelial cells of secretors and non-secretors.**

## Staphylococcus aureus Strain 10654



**Figure 3.14** Binding of *S. aureus* (NCTC 10654) to epithelial cells of secretors and non-secretors.

## Staphylococcus aureus Strain 10656



**Figure 3.15** Binding of *S. aureus* (NCTC 10656) to epithelial cells of secretors and non-secretors.

## Staphylococcus aureus Strain 10655

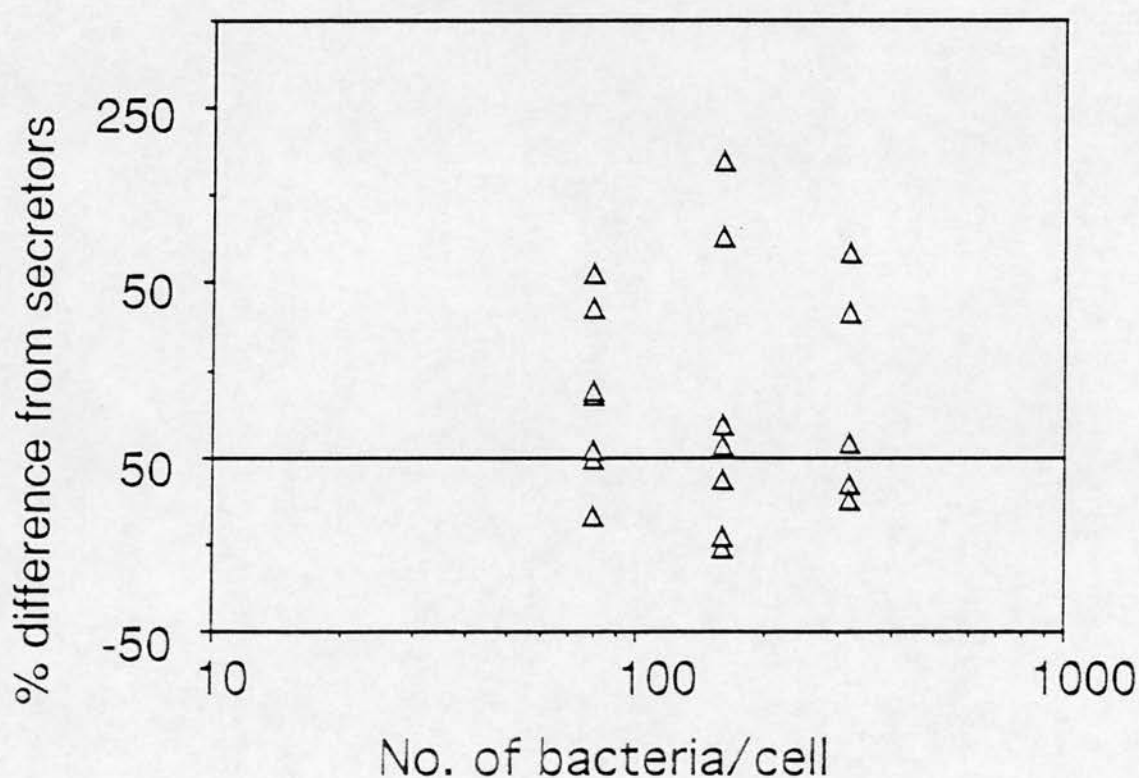


Figure 3.16 Binding of *S. aureus* (NCTC 10655) to epithelial cells of secretors and non-secretors.

## Staphylococcus aureus Strain 10657

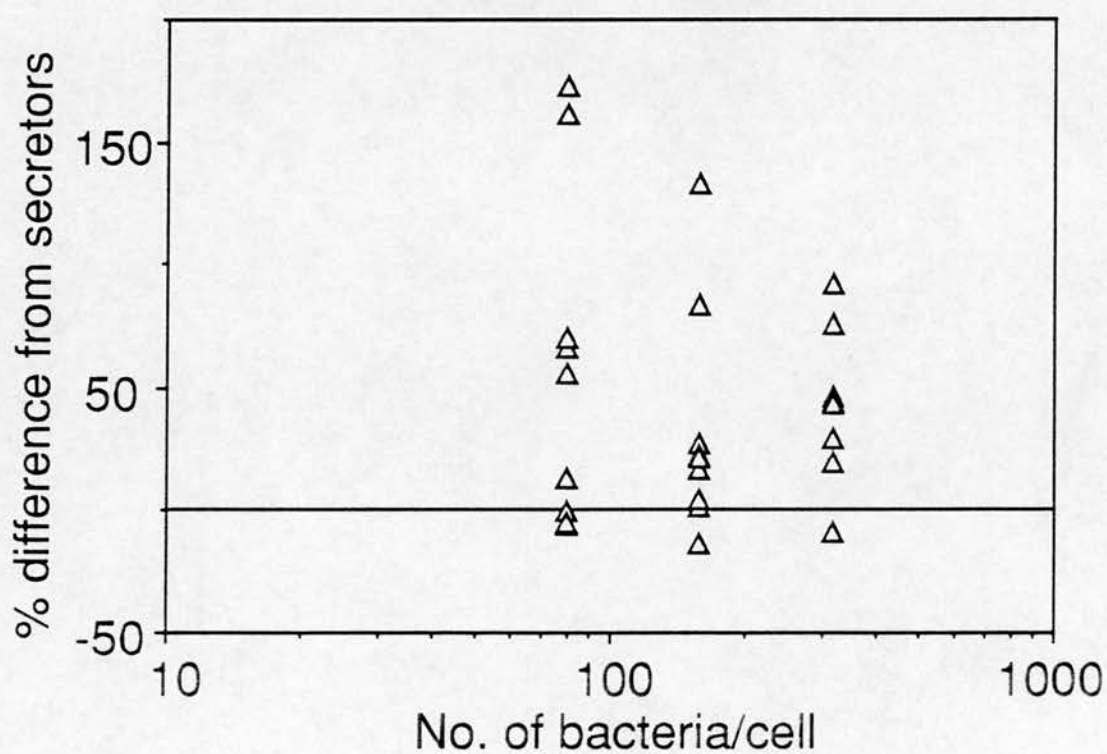
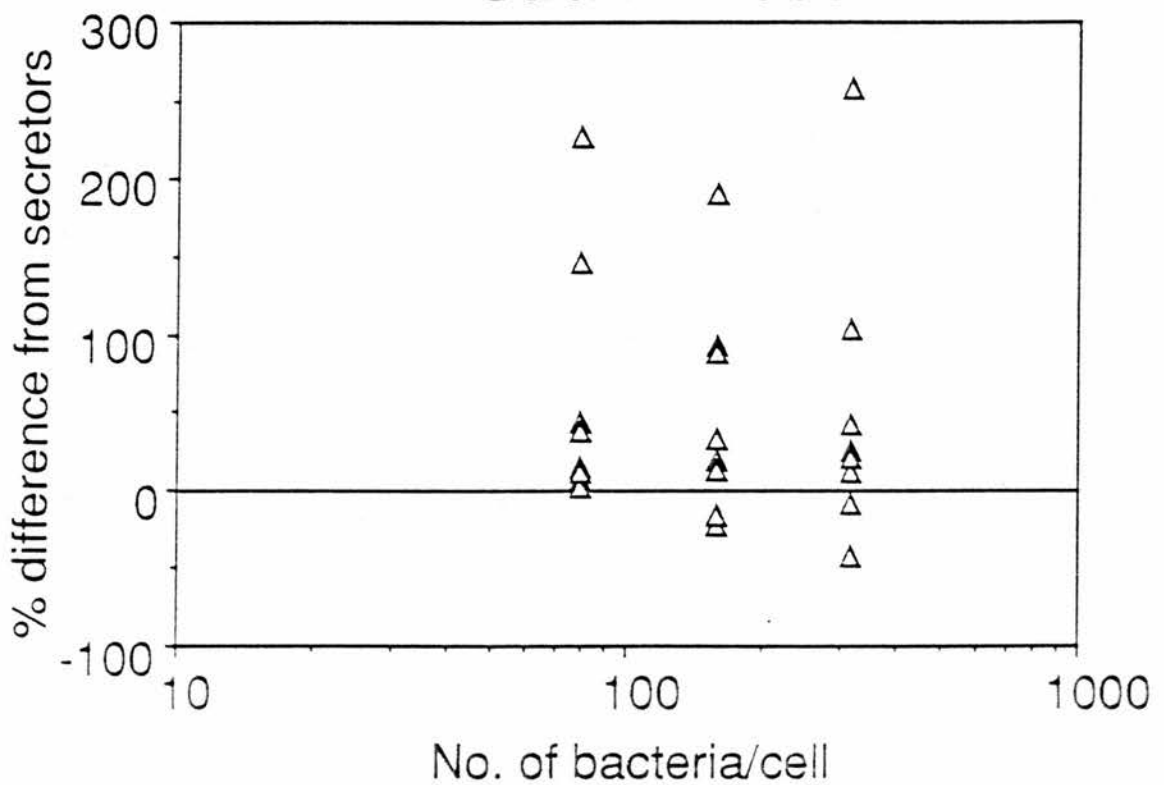


Figure 3.17 Binding of *S. aureus* (NCTC 10657) to epithelial cells of secretors and non-secretors.

## Staphylococcus aureus Strain 11965



**Figure 3.18 Binding of *S. aureus* (NCTC 11965) to epithelial cells of secretors and non-secretors.**

**Table 3.3 95% confidence limits for binding of *S. aureus* to non-secretor cells expressed as a percentage of binding to cells from matched secretors.**

| <u>Strain</u> | <u>80 Bacteria : cell</u> | <u>160 Bacteria : cell</u> | <u>320 Bacteria : cell</u> |
|---------------|---------------------------|----------------------------|----------------------------|
| NCTC 8532     | 66 - 145                  | 69 - 146                   | 31 - 182                   |
| NCTC 10652    | 63 - 198                  | 63 - 227                   | 50 - 301                   |
| NCTC 10654    | 67 - 153                  | 75 - 185                   | 75 - 206                   |
| NCTC 10655    | 138 - 228**               | 107 - 249*                 | 107 - 237*                 |
| NCTC 10656    | 61 - 136                  | 79 - 175                   | 67 - 178                   |
| NCTC 10657    | 110 - 197*                | 101 - 161*                 | 117 - 165**                |
| NCTC 11965    | 102 - 201*                | 92 - 192                   | 81 - 203                   |
| 40654         | 82 - 182                  | 54 - 189                   | 91 - 222                   |
| 41206         | 74 - 147                  | 75 - 172                   | 74 - 158                   |

\*  $p < 0.05$ ; \*\*  $p < 0.01$

### 3.4 Discussion

The objective of this study was to test the hypothesis that epithelial cells from non-secretor donors bind greater numbers of toxigenic staphylococci than cells from secretors. BEC were chosen for the study of the binding assays because of their ready availability and their interaction with bacteria will mimic the natural system *in vivo*. Although many cells are dead and there are variations in size of the cells, they have been used for binding assays with many bacterial species: type III Group-B *Streptococci pyogenes* [Bagg, 1982], *Neisseria meningitides* [Craven and Frasc, 1978] and *S. aureus* [Beck, 1989].

A number of different techniques were used to measure the attachment of bacteria to cells. There were several problems associated with the light microscopy method for assessment of bacterial binding. Bacteria were not evenly distributed over the BEC. There were background commensal organisms which affected the counting of bound bacteria and the number of bacteria observed varied greatly. Similar variability has been observed by Anderson *et al.* [1981]. It is a very laborious experiment because at least 40 cells must be counted for each test or control sample. CSOM was useful to confirm the bacteria were bound to cells. Although the FITC dye distinguished the staphylococcal test strain from commensal bacteria, it is time consuming to do the counting for a reasonable number of cells, and CSOM is an expensive technique.

ELISA was used to detect bacteria bound to epithelial cells. Both the FITC-labelled and un-labelled bacteria were used. The OD values for both the labelled and the un-labelled cells were close, but there was a high OD reading for the control to which no bacteria had been added, probably due to anti-toxin binding to molecules on BEC or to the commensal bacteria. When the background was subtracted, there appeared to be a



correlation between the number of bacteria added and the OD reading for both FITC labelled and unlabelled *S. aureus*.

Flow cytometry provided a potentially powerful tool for analysing bacterium-cell interactions. It is a reliable and practical method. Large numbers of cells were analysed in a short period of time. Dyes such as fluorescein isothiocyanate (FITC) that bind covalently to protein amino groups are preferable to other acid dyes because washing lowers background fluorescence [Shapiro, 1990]. Direct labelling of bacteria with FITC was used for adhesion assays by other workers [Svenson and Kallenius 1983]. Direct labelling of *S. aureus* with FITC was the method chosen for these studies. Rhodamine 123 which binds to the mitochondria has been used to stain bacteria [Kaprelyants, 1992]. The binding indices obtained with FITC-labelled *S. aureus* were much higher than those obtained with rhodamine 123 (Fig 3.5). The binding of *S. aureus* to BEC was rapid and saturable. There was adequate binding at 30 min (Fig 3.6), and the binding was clear at ratios of 80-640 bacteria / cell. There was not much variation in the binding index when different ratios of 4 strains of *S. aureus* were used for binding with cells from one donor (Fig 3.8).

Non-secretors of ABO blood group antigens are over-represented among carriers of group A streptococci [Haverkorn and Goslings 1969], and because density of colonization might be an important consideration in the hypothesis that toxin producing bacteria play a role in some cot deaths we tested the hypothesis that epithelial cells from non-secretor donors bind greater numbers of toxigenic staphylococci than cells from secretors. The results in Table 3.3 indicated variations in the results of binding indices among both the secretors and the non-secretors. The source of BEC was dependent on the availability of the individuals during the day of the test; therefore, BEC were not always from the same individuals for the various strains tested. The results also indicated that 3 of the 8 toxigenic strains, including one

producing TSST-1, bound in significantly greater numbers to non-secretor cells (Fig 3.16, 3.17 and 3.18). The 5 remaining toxigenic strains and the non-toxigenic strain showed no difference in binding to cells of secretors compared with non-secretors (Fig 3.10, 3.11, 3.12, 3.13, 3.14 and 3.15).

The results indicated either that different individuals express different amount of Le<sup>a</sup> on their epithelial cells or the adhesins which recognise Le<sup>a</sup> molecules are found only on the 3 strains which showed the higher binding to cells from non-secretors. These two possibilities are the subject of the next chapter.

## Chapter 4

# Determination of Lewis<sup>a</sup> antigen on BEC and its possible role as a binding site to *S. aureus* adhesins

### 4.1 Introduction

The results in chapter 3 indicated that only three strains of toxigenic *S. aureus* bound to cells from non-secretors in greater quantities than cells from secretors, hence two explanations were proposed. First, there might be variable amounts of Le<sup>a</sup> on the cells of secretors. Second the adhesins that bind Le<sup>a</sup> were present only on the three strains which bound more to cells from non-secretors.

Non-secretors can produce only Le<sup>a</sup> while secretors produce predominantly Le<sup>b</sup> and also variable amount of Le<sup>a</sup> [Race and Sanger, 1968]. The production of Lewis substances is due to the competition between two fucosyl transferases, one coded for by the secretor gene (*Se*) and one coded for by the Lewis gene (*Le*). Both these enzymes add fucose to the type 1 precursor chain from which most of the ABO and Lewis antigens in secretions are derived (Chapter 1). If the secretor transferase adds fucose to the terminal sugar in the precursor chain, the Lewis enzyme can add fucose to the sub terminal sugar to produce Le<sup>b</sup>. If the Lewis enzyme adds fucose to the sub-terminal sugar first to produce Le<sup>a</sup>, the secretor enzyme cannot use this structure as a substrate and Le<sup>a</sup> is the final product [Watkins *et al.*, 1988]. The relative amounts of Le<sup>a</sup> present in a secretor will depend on the efficiencies of the two enzymes. If the secretor enzyme is more efficient, most of the type 1 precursor chain will be present as Le<sup>b</sup>. If the Le<sup>a</sup> enzyme is more efficient, there will be relatively more Le<sup>a</sup>. Non-

secretors can produce only Le<sup>a</sup> as they lack the transferase coded for by the secretor gene.

May *et al.* [1989] have shown that pre-treatment of BEC of a non-secretor donor with monoclonal anti-Le<sup>a</sup> will significantly inhibit the binding with candida blastospores. If the adhesins that bind Le<sup>a</sup> were present only on those strains that bound in greater amounts to cells of non-secretors, it was predicted that their binding to BEC could be reduced by pre-treatment of the cells with monoclonal anti-Le<sup>a</sup>. Binding of strains that did not express the Le<sup>a</sup> adhesin (e.g. NCTC 8532) would be unaffected by pre-treatment of the cells with the antibody.

The objectives of this part of the study were:

1. To assess levels of Le<sup>a</sup> on BEC of secretors and non-secretors by detection of binding of monoclonal anti-Le<sup>a</sup> to the cells.
2. To test the hypothesis that attachment of *S. aureus* NCTC 10655 that bound in greater numbers to cells of non-secretors could be reduced by pre-treatment of the cells with anti-Le<sup>a</sup>, but that binding of NCTC 8532 would not be affected by this treatment.

## **4.2 Materials and methods**

### **4.2.1 Detection of Le<sup>a</sup> and Le<sup>b</sup> on erythrocytes**

The Lewis red cell phenotype of all secretors and non-secretors tested was determined by tube agglutination test as described in (2.6)

#### **4.2.2 Detection of Le<sup>a</sup> and Le<sup>b</sup> antigens in saliva of secretors and non-secretors**

An ELISA was used to detect Le<sup>a</sup> and Le<sup>b</sup> blood group antigens in saliva. Synsorb purified monoclonal anti-Le<sup>a</sup> diluted 1/25 (100 µl) or anti-Le<sup>b</sup> diluted 1/10 (100 µl) in coating buffer was added to wells of polystyrene microtitre plates (Dynatech, Billingham, Sussex) and incubated overnight at 4°C. The wells were washed 3 times in washing buffer and blocked by 100 µl of blocking buffer. The plates were washed 3 times and 100 µl of each saliva specimen diluted 1/10 in PBS were added to the wells (At 1/10 dilution of saliva, the absorbance was higher than with the neat saliva because saliva contains mucus and other proteins). The plates were incubated at RT for 60 min and washed 3 times. Polyclonal goat anti-Le<sup>a</sup> antibody (Behring, 100 µl diluted 1/25) or polyclonal goat anti-Le<sup>b</sup> antibody (Behring, 100 µl diluted 1/25) was added to the appropriate wells and incubated for 60 min. The plates were washed and 100 µl of horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (1/250) were added for 2h at RT. The plates were washed and 50 µl of the substrate (section 2.1.6) were added. After incubation for 20 min at RT, the reaction was stopped by adding 50 µl of stopping solution (section 2.1.7) to each well. Absorbance at 490 nm was measured with a Dynatech plate reader. The readings for duplicate samples were averaged.

#### **4.2.3 Detection of Le<sup>a</sup>, Le<sup>b</sup>, type 1 precursor and Le<sup>x</sup> antigens on BEC of secretors and non-secretors**

BEC (200 µl,  $2 \times 10^5$  ml<sup>-1</sup>) were added to Falcon tubes containing 200 µl of monoclonal antibodies to Le<sup>a</sup> (1/5, SAPU), Le<sup>b</sup> (1/10, SAPU), precursor-type 1 (1/10, Russel Fine Chemicals) or Le<sup>x</sup> (1/10, serotec). The tubes were incubated at RT for 60 min, washed twice with PBS at 300 g for 10 min and incubated with 200 µl rabbit anti-mouse IgM conjugated with FITC (1/200, sigma). The FITC-labelled antibody was

also added to 200  $\mu$ l of cells which had not been treated with the first antibody as a control. The tubes were incubated at 37°C for 60 min with continuous shaking. The cells were washed twice with PBS and fixed with 200  $\mu$ l of 1% buffered paraformaldehyde and stored in the dark at 4°C until analysed. The cells were analysed on an EPICS-C flow cytometer (3.2.3.4).

#### **4.2.4 BEC adsorption of Le<sup>a</sup> from non-secreter saliva**

Serial dilutions (200  $\mu$ l) of fresh unboiled saliva from a non-secreter were added to 200  $\mu$ l of a suspension of BEC ( $2 \times 10^5$  cells / ml) from a Le<sup>a-b-</sup> individual and from a non-secreter individual. Neat saliva (200  $\mu$ l) from the Le<sup>a-b-</sup> donor was added to cells as a control. The effect of boiled saliva was also tested. The tubes were incubated at RT for 60 min, washed twice in PBS and the binding of anti-Le<sup>a</sup> to the treated and untreated cells detected by the flow cytometer method described in 4.2.3.

#### **4.2.5 Inhibition of bacterial binding by pre-treatment of BEC with monoclonal anti-Le<sup>a</sup>, anti-Le<sup>x</sup> or anti-precursor type 1**

BEC obtained from secretors and non-secretors (200  $\mu$ l) were incubated with 200  $\mu$ l of monoclonal anti-Le<sup>a</sup> (SAPU), anti-precursor type 1 (Russel Fine Chemicals) or anti-Le<sup>x</sup> (Serotec) at 37°C for 60 min. After washing twice by centrifugation at 300 g for 10 min, the attachment assays with FITC-labelled NCTC 10655 and NCTC 8532 were performed and analysed as described in (3.2.3.4).

#### **4.2.6 Statistical methods**

All analyses were carried out on the logarithms of the binding indices which conformed more closely to a normal distribution than the raw values. Differences between groups

were tested by paired or un-paired *t* tests as appropriate, and confidence limits for the mean values in one group were expressed as a percentage of those in the other by taking antilogarithms. Analysis of covariance was used to test whether binding levels were associated with the amount of anti-Le<sup>a</sup> in secretors and non-secretors tested on different days, while the association in a similar experiment with anti-precursor type 1 on a single day was tested by the Pearson correlation.

## **4.3 Results**

### **4.3.1 Detection of Le<sup>a</sup> and Le<sup>b</sup> on erythrocytes**

The secretor status of the donors was determined by the haemagglutination inhibition assays. The results of tube agglutination tests were positive with anti-Le<sup>a</sup> and negative with anti-Le<sup>b</sup> with erythrocytes from individuals classed as non-secretors by the haemagglutination inhibition assay with their saliva. Cells from secretors were agglutinated by anti-Le<sup>b</sup> except for two secretors (out of 12 donors) whose cells were not agglutinated by either anti-Le<sup>a</sup> or anti-Le<sup>b</sup>.

### **4.3.2 Detection of Le<sup>a</sup> and Le<sup>b</sup> in saliva by ELISA**

Uniformly high readings were obtained with the saliva of nine non-secretors in the ELISA for Le<sup>a</sup> antigen, with OD ranging from 1.322 to 1.752. The OD for Le<sup>b</sup> values were low (< 0.1) in all except for one which was (0.180) (Fig 4.1). There were variable results for both Le<sup>a</sup> and Le<sup>b</sup> in saliva of 10 secretors whose erythrocytes were agglutinated by anti-Le<sup>b</sup>. There were very low OD readings (< 0.050) for both Le<sup>a</sup> and Le<sup>b</sup> with saliva from donors 1 and 2 whose erythrocytes were not agglutinated with either antibody (Fig 4.2).



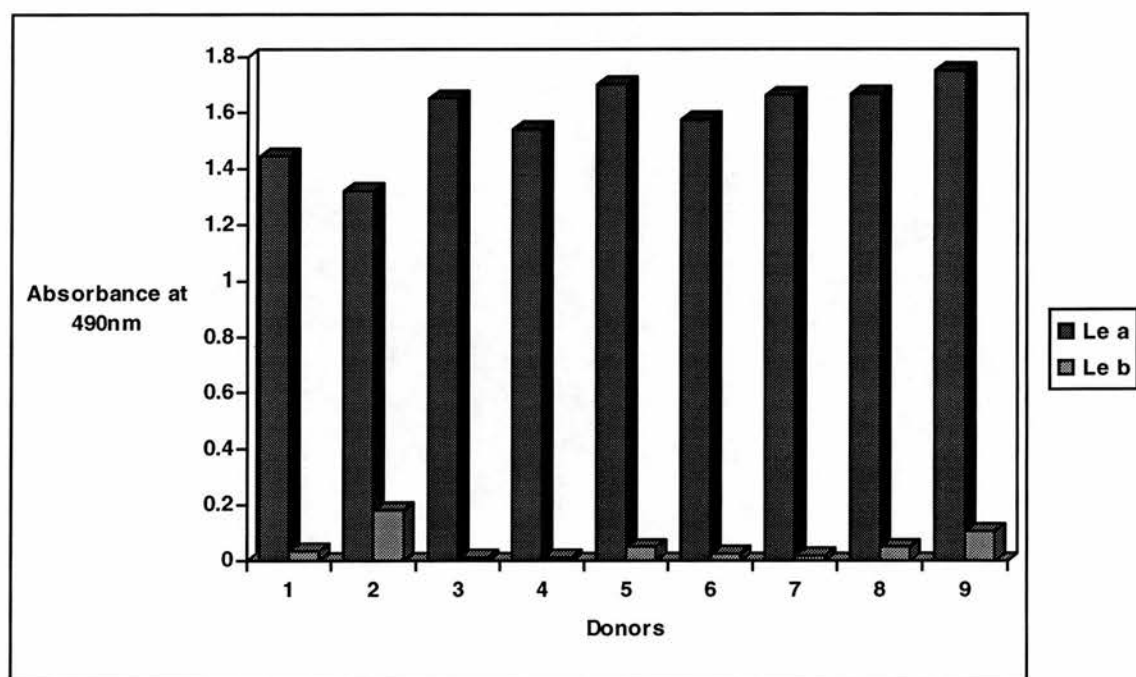


Figure 4.1 OD values for detection of Le<sup>a</sup> and Le<sup>b</sup> in saliva of non-secretors.



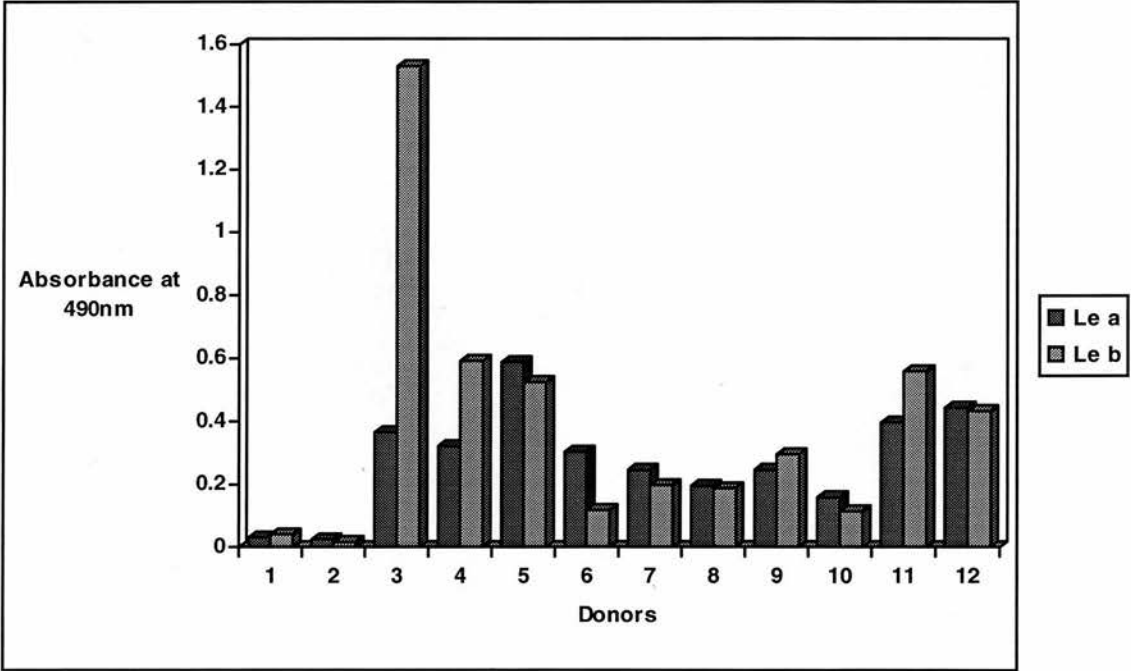


Fig 4.2 OD values for detection of Le<sup>a</sup> and Le<sup>b</sup> in saliva of secretors.

### **4.3.3. Detection of Le<sup>a</sup> on BEC**

#### **4.3.3.1 Titration of FITC anti-mouse antibody and of anti-Le<sup>a</sup>**

Serial dilutions of FITC anti-mouse IgM in PBS (200 µl) were added to BEC (200 µl) to choose the appropriate concentration at which non-specific binding was minimal. The 1/200 dilution showed low fluorescence readings and was used for the assay.

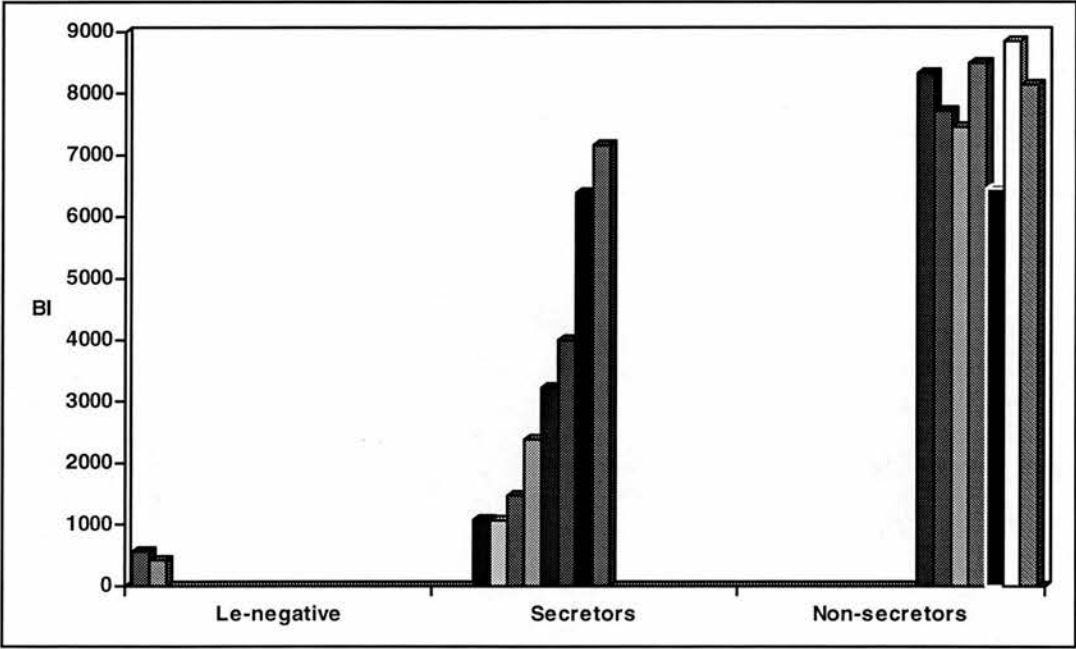
The optimal dilution of anti-Le<sup>a</sup> was determined by the method described in (4.2.3) with serial dilutions of anti-Le<sup>a</sup>. The 1/5 dilution was chosen for the assays as a high percentage of cells of non-secretors were positive compared with much lower readings for cells from Lewis negative donors (Table 4.1).

#### **4.3.3.2 Binding of anti-Le<sup>a</sup> to buccal epithelial cells of secretors and non-secretors**

The buccal epithelial cells (BEC) from the individuals whose erythrocytes were not agglutinated by either anti-Le<sup>a</sup> or anti-Le<sup>b</sup> (Le<sup>a-b-</sup> or Le-negative) bound low levels of anti-Le<sup>a</sup>. Cells from non-secretors bound high levels of the antibody, both in the percentage and mean values. While the mean binding index for secretors (36707) was approximately half that for non-secretors (79148), there was considerable variation in the amount of antibody bound by individual secretors, both in the percentage and mean values (Fig 4.3). The average value for binding index for a non-secretor was 16 times more than for Le<sup>a-b-</sup> individual and this low value could be non-specific.

**Table 4.1 Titration of anti-Le<sup>a</sup> for the BEC assays**

| <u>Donor</u>                   | <u>Anti-Lewis<sup>a</sup></u> | <u>fluorescence reading</u> |             |
|--------------------------------|-------------------------------|-----------------------------|-------------|
|                                | <u>Dilution</u>               | <u>%</u>                    | <u>mean</u> |
| Non-secretor                   | 1/5                           | 92.3                        | 44.8        |
|                                | 1/10                          | 80.6                        | 31.6        |
|                                | 1/50                          | 43.6                        | 10.4        |
| Secretor (Le <sup>a-b-</sup> ) | 1/5                           | 25.2                        | 20.6        |
|                                | 1/10                          | 3.7                         | low         |



**Figure 4.3 Binding of monoclonal anti-Lewis<sup>a</sup> to BEC from 2 Le-negative secretor, 8 secretor and 7 non-secretor donors.**

#### **4.3.4 Binding of anti-Lewis<sup>b</sup> to BEC**

Serial dilutions of anti-Le<sup>b</sup> in PBS were used to detect Le<sup>b</sup> on BEC. At the 1/10 dilution, the percentage of binding obtained with cells of a secretor donor was high; however at this concentration there was significant fluorescence with cells from a non-secretor (Fig 4.4). The cross reactivity was more obvious when BEC from four secretors, four non-secretors and two Le<sup>a-b-</sup> were tested for binding of anti-Le<sup>b</sup> (Fig 4.5)

#### **4.3.5 Binding of anti-precursor type 1 to BEC**

The optimal dilution for the monoclonal anti-precursor type 1 was 1/10 (Fig 4.6) and it was used for further experiments. There was no consistent pattern of binding of the anti-precursor monoclonal to cells of secretors, non-secretors or Lewis-negative individuals (Fig 4.7).

#### **4.3.6 Binding of anti-Lewis<sup>x</sup> to BEC**

The optimal dilution for monoclonal anti-Le<sup>x</sup> was 1/10 (Fig 4.8). The binding indices for anti-Le<sup>x</sup> were lower than those for anti-Le<sup>a</sup> when tested with cells from the same donors. There was no correlation between binding of anti-Le<sup>x</sup> and anti-Le<sup>a</sup> to cells from the same non-secretor donors (Fig 4.9).

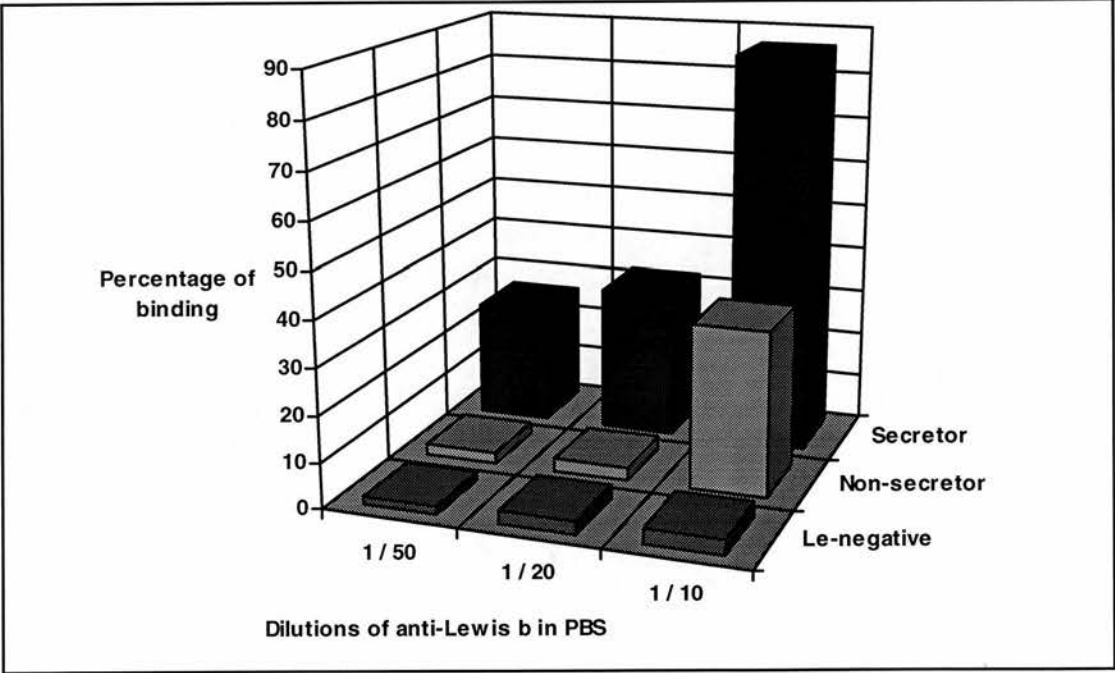


Figure 4.4. Titration of anti-Lewis<sup>b</sup> against BEC of secretor, non-secretor and Lewis negative donor.

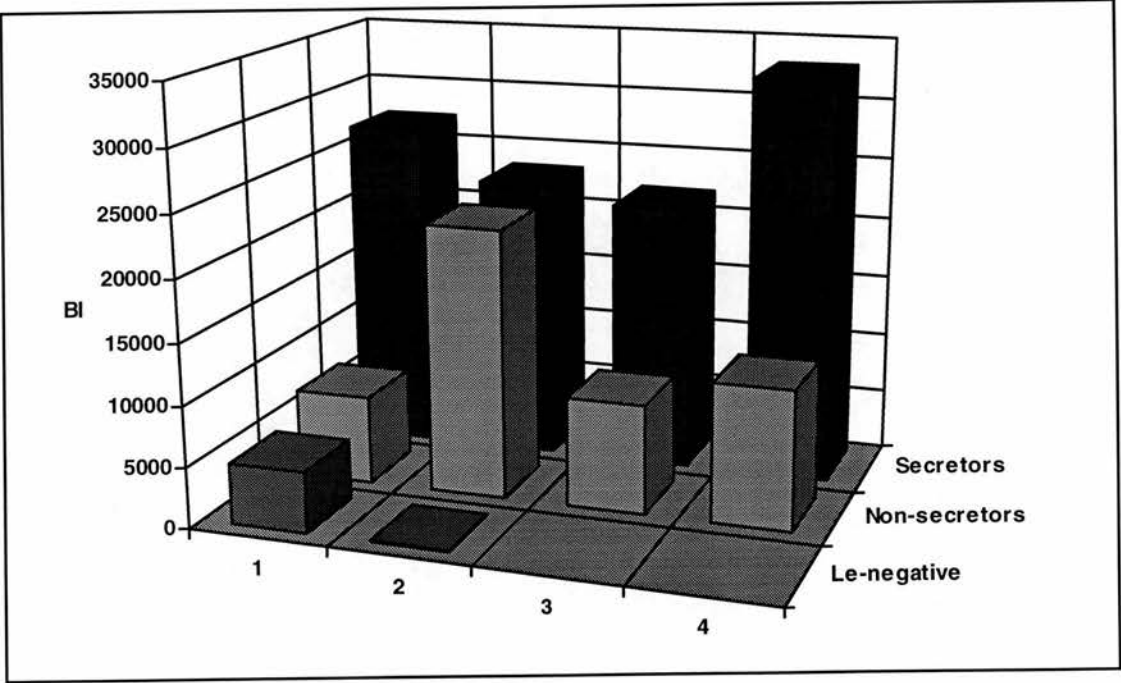
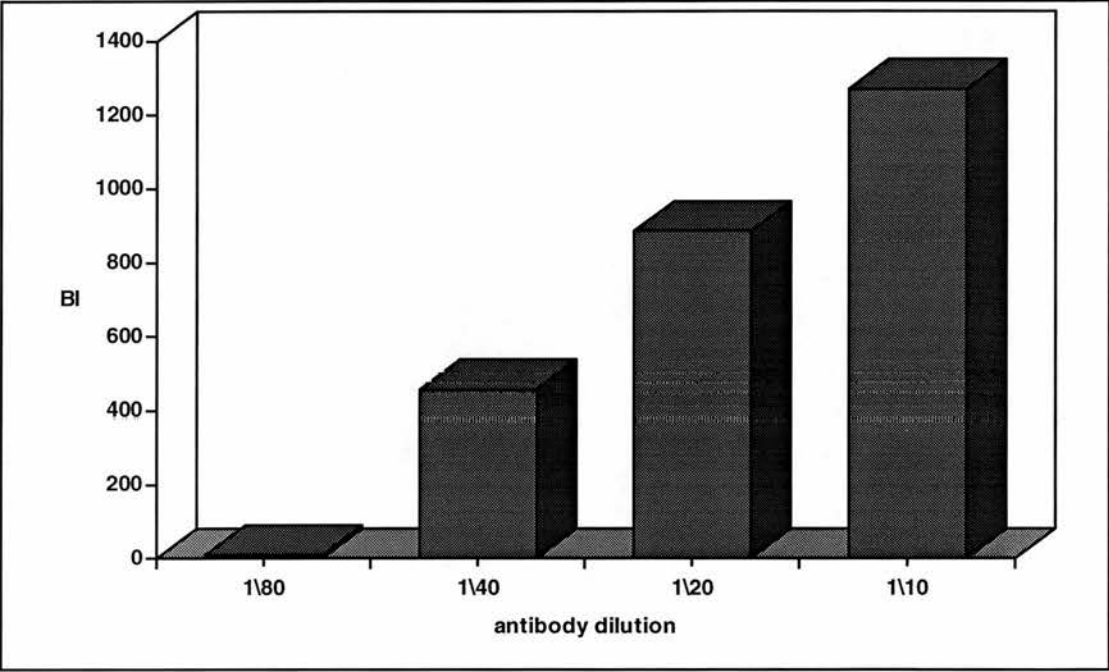
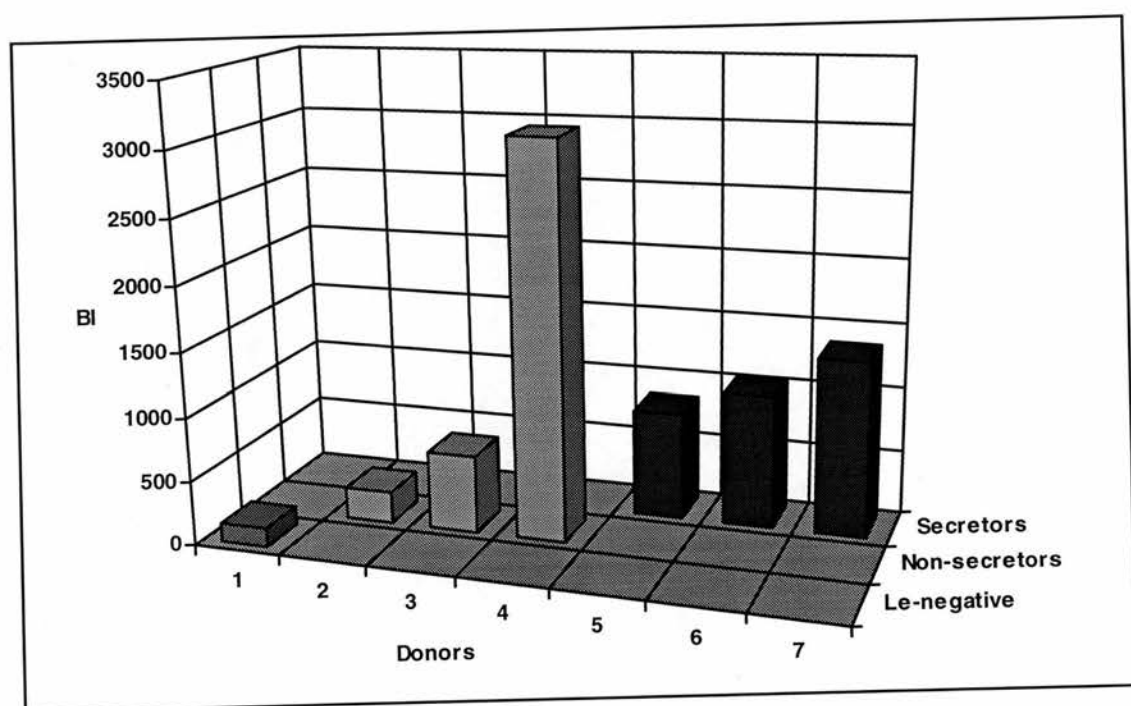


Figure 4.5 Binding of monoclonal anti-Lewis<sup>b</sup> (1/10) to BEC from 2 Lewis negative secretors, 4 non-secretors and 4 secretor donors.

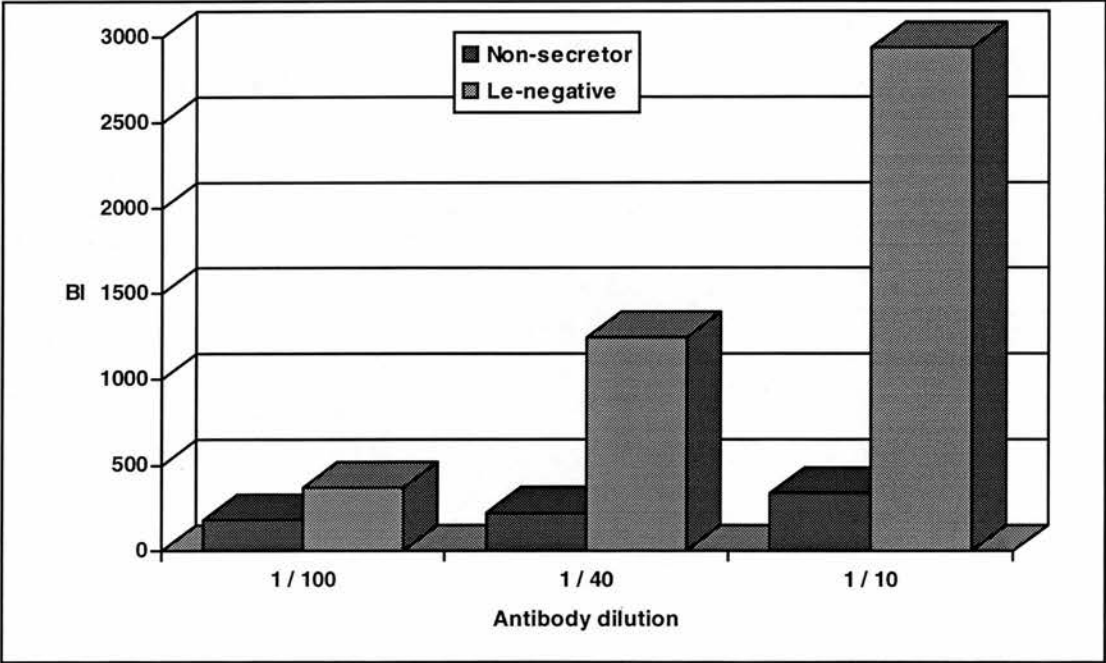


**Figure 4.6 Titration of anti-precursor type 1 against BEC from a non-secretor donor.**





**Figure 4.7 Binding of monoclonal anti-precursor type 1 (1/10) to BEC of Le negative, non-secretor or secretor donors.**



**Figure 4.8 Titration of monoclonal anti-Le<sup>x</sup> against BEC of a non-secretor and a Le-negative secretor donor.**

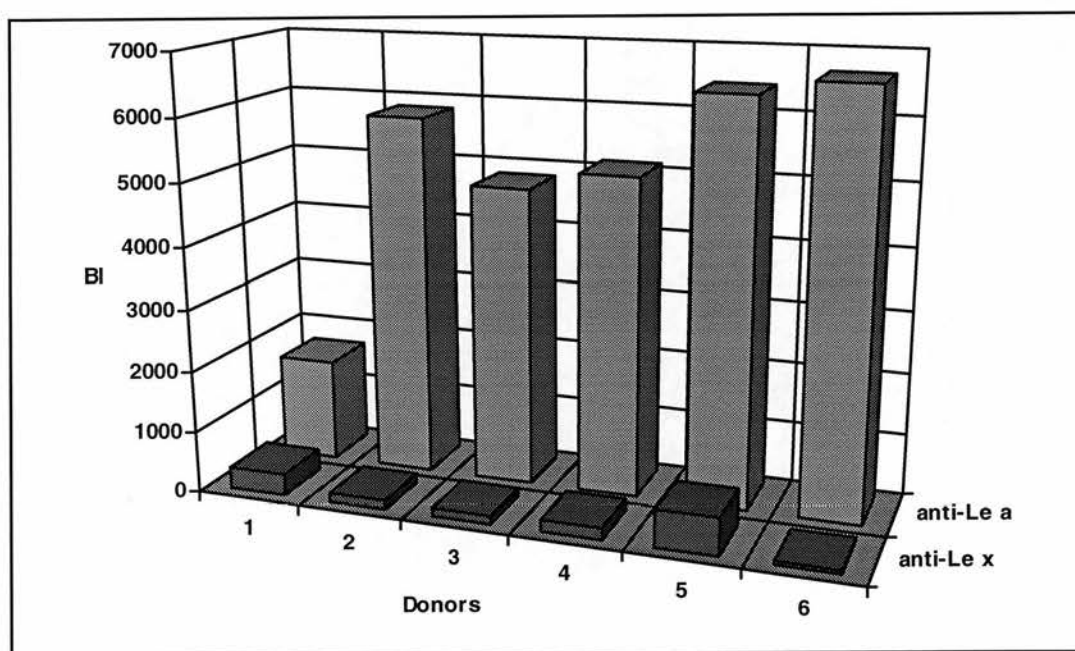


Figure 4.9 Binding indices for anti-Le<sup>x</sup> and anti-Le<sup>a</sup> to BEC of non-secretors.

### 4.3.7 Adsorption of $Le^a$ by BEC of a $Le^a-b^-$ donor

The flow cytometry assay (section 3.2.3.4) was used to detect anti- $Le^a$  bound to cells treated with saliva from  $Le^{a+b^-}$  and  $Le^{a-b^-}$  donors.

Adding saliva of a non-secretor ( $Le^{a+b^-}$ ) to BEC of a Le-negative individual ( $Le^{a-b^-}$ ) increased the binding index of anti- $Le^a$  and the increase was dose dependent Table 4.2. Table 4.3 shows the effect of adding saliva from 7 non-secretors and saliva from a  $Le^{a-b^-}$  donor to BEC of a  $Le^{a-b^-}$  individual. Generally adding saliva from non-secretors increased the binding indices; however, there were variation in the values obtained by using saliva of different individuals. ELISA technique have shown non-secretors expressed high levels of  $Le^a$ ; however, this variation in the uptake of  $Le^a$  could be due to the variation in the availability of free  $Le^a$  molecules in the saliva of different individuals. Adding saliva from a  $Le^{a-b^-}$  individual increased the binding index much less than saliva from the non-secretors.

The effect of adding saliva of a non-secretor to BEC of a non-secretor was much less compared with its effect on BEC of a Le-negative individual, while adding saliva from a  $Le^{a-b^-}$  individual to BEC from either donor resulted in a much smaller increase (Table 4.4). Both the percentage and the mean for  $Le^{a-b^-}$  was low as compared to those for  $Le^{a+b^-}$ . The main difference was in the mean. The mean value for  $Le^{a+b^-}$  was 7 times more than that for  $Le^{a-b^-}$ . By adding  $Le^{a-b^-}$  saliva to homologous cells the mean did not change. In contrast, adding saliva from  $Le^{a+b^-}$  to  $Le^{a-b^-}$  cells the mean increased 17 times.

**Table 4.2 Binding of anti-Le<sup>a</sup> to cells from a Lewis negative donor treated with PBS, homologous saliva or non-secretor saliva.**

| <u>treatment</u>                              | <u>Binding index</u> |
|---|----------------------|
| PBS   | 1376                 |
| Le <sup>a</sup> -b <sup>-</sup> saliva (Neat) | 1527                 |
| Le <sup>a</sup> +b <sup>-</sup> saliva (Neat) | 3575                 |
| 1 / 10  | 2986                 |
| 1 / 100                                       | 1824                 |

**Table 4.3 Binding of anti-Le<sup>a</sup> to cells from a Lewis negative donor treated with PBS, homologous saliva and saliva from 7 non-secretors.**

| <u>Treatment</u>                       | <u>binding index</u> |
|--|----------------------|
| PBS                                    | 3229                 |
| Le <sup>a</sup> -b <sup>-</sup> saliva | 4353                 |
| Le <sup>a</sup> +b <sup>-</sup> saliva |                      |
| 1                                      | 5234                 |
| 2                                      | 6218                 |
| 3                                      | 8370                 |
| 4                                      | 12200                |
| 5                                      | 15351                |
| 6                                      | 20178                |
| 7                                      | 22860                |

**Table 4.4 Binding of anti-Le<sup>a</sup> to cells from Le<sup>a</sup>-b<sup>-</sup> or a non-secretor donor treated with PBS, saliva from a Le<sup>a</sup>-b<sup>-</sup> or a Le<sup>a</sup>+b<sup>-</sup> donor.**

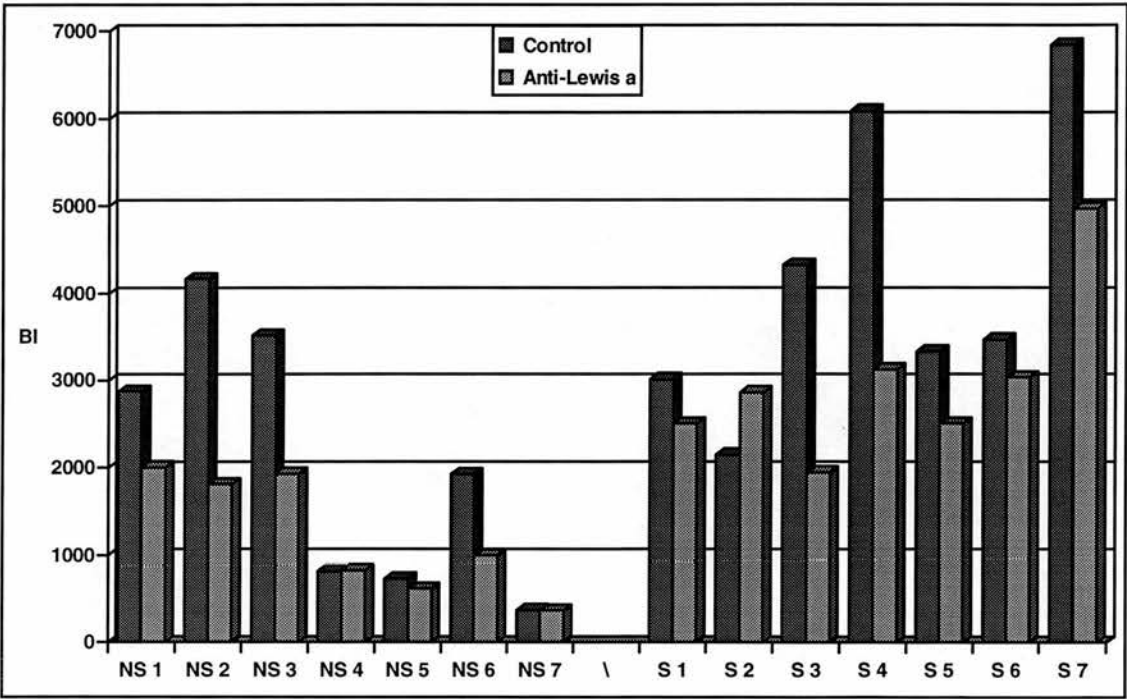
| <u>Cells</u>   | <u>Treatment</u> | <u>percentage</u> | <u>mean</u> | <u>Binding index</u> |
|--|------------------|-------------------|-------------|----------------------|
| Le <sup>a</sup> -b <sup>-</sup>  | + PBS            | 22.6              | 25.6        | 579                  |
| Le <sup>a</sup> -b <sup>-</sup> cells + Le <sup>a</sup> -b <sup>-</sup> saliva |                  | 51.2              | 25          | 1280                 |
| Le <sup>a</sup> -b <sup>-</sup> cells + Le <sup>a</sup> +b <sup>-</sup> saliva |                  | 91.4              | 440         | 40216                |
| Le <sup>a</sup> +b <sup>-</sup> cells + PBS                                    |                  | 89.2              | 178         | 15878                |
| Le <sup>a</sup> +b <sup>-</sup> cells + Le <sup>a</sup> -b <sup>-</sup> saliva |                  | 97.3              | 175         | 17028                |
| Le <sup>a</sup> +b <sup>-</sup> cells + Le <sup>a</sup> +b <sup>-</sup> saliva |                  | 96.5              | 190         | 18335                |

### 4.3.8 Inhibition of bacterial binding

Binding of *S. aureus* NCTC 10655 to BEC obtained from 7 secretors and 7 non-secretors treated with anti-Le<sup>a</sup> antibody was significantly lower compared with binding of the bacteria to untreated cells from the same donors ( $t = 4.46$ , d.f. = 13,  $P < 0.001$ , 95 % CI 55-81) (Fig 4.10). The inhibition was not 100% which implies that there are other receptors involved in the binding. A similar pattern was observed for binding of NCTC 10655 to cells of 7 non-secretors and 10 secretors treated with monoclonal anti-precursor type 1 compared with untreated cells ( $t = 8.19$ , d.f. = 16,  $P < 0.001$ , 95 % CI 58-72) (Fig 4.11). Binding of this strain to BEC was significantly reduced when the cells from 6 non-secretor donors were treated with anti-Le<sup>x</sup> compared with binding of untreated cells (Fig 4.12).

Although there was no difference in binding of NCTC 8532 to BEC of non-secretors compared to secretors, binding of NCTC 8532 to the cells from 7 non-secretor and 6 secretor donors treated with anti-Le<sup>a</sup> antibody was significantly lower compared with binding of the bacteria to untreated cells ( $t = -4.97$ , d.f. = 11,  $p < 0.001$ , 95 % CI 43-72) Fig (4.13). This implies that the difference in bacterial binding is more likely to the difference in the expression of antigens on the BEC. Inhibition of NCTC 8532 with anti-precursor type 1 or anti-Le<sup>x</sup> was not examined because of the limited amount of the antibodies.





**Figure 4.10** Effect of incubation of anti-Lewis<sup>a</sup> with BEC from non-secretor (NS) and secretor donors (S) on binding of *S. aureus* NCTC 10655.

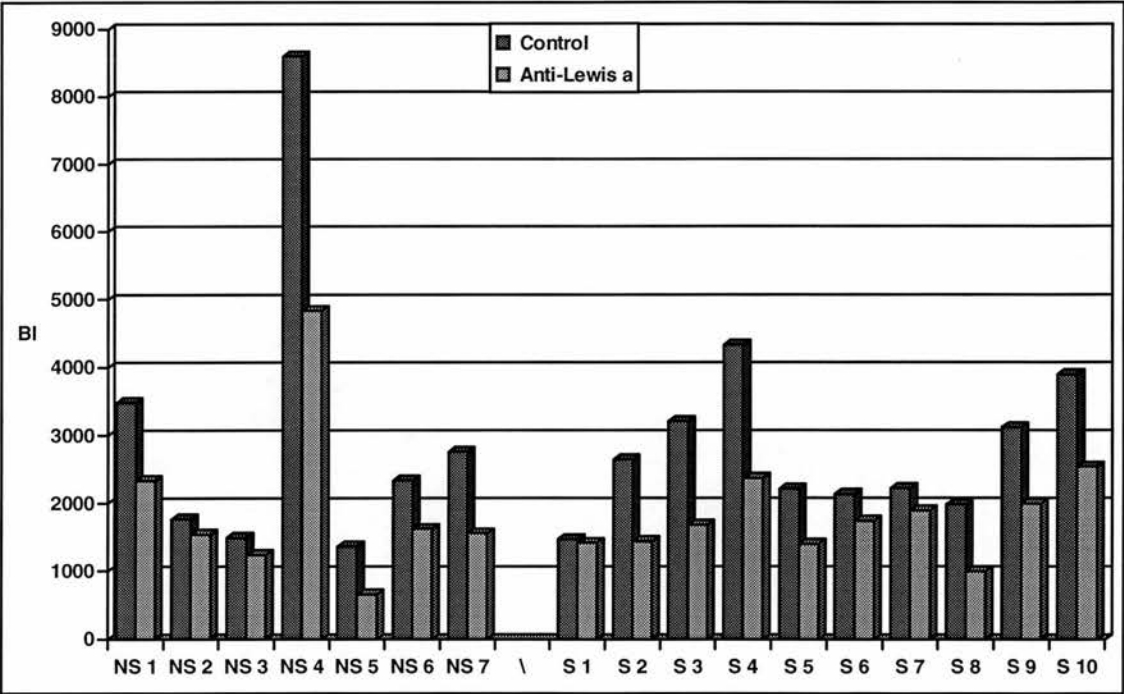
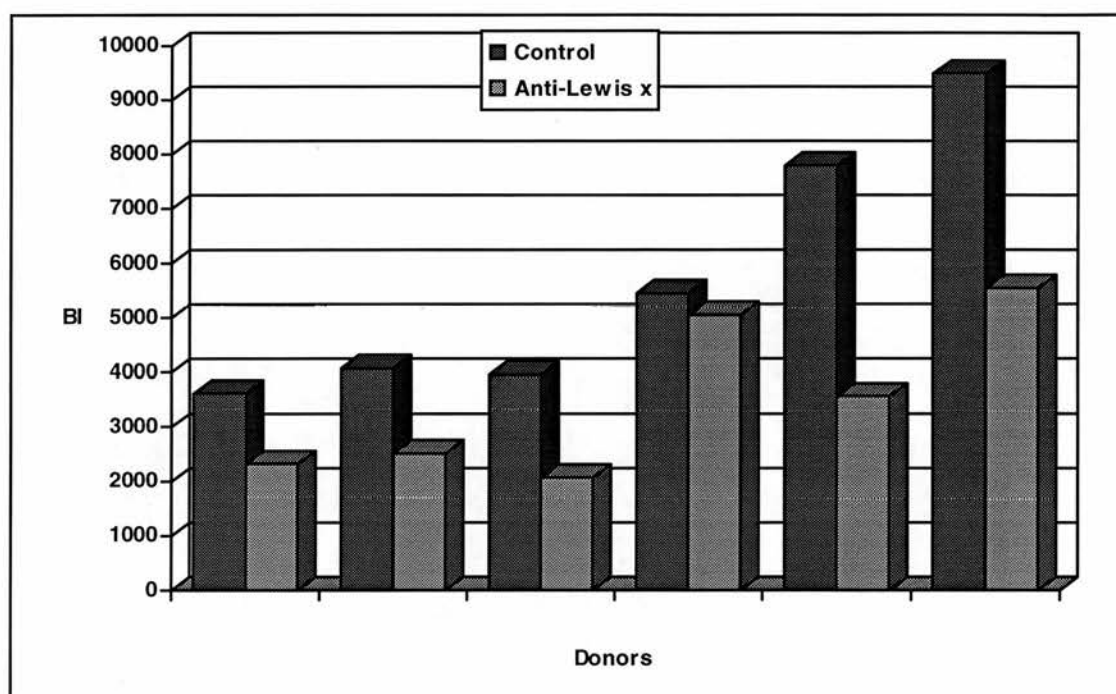
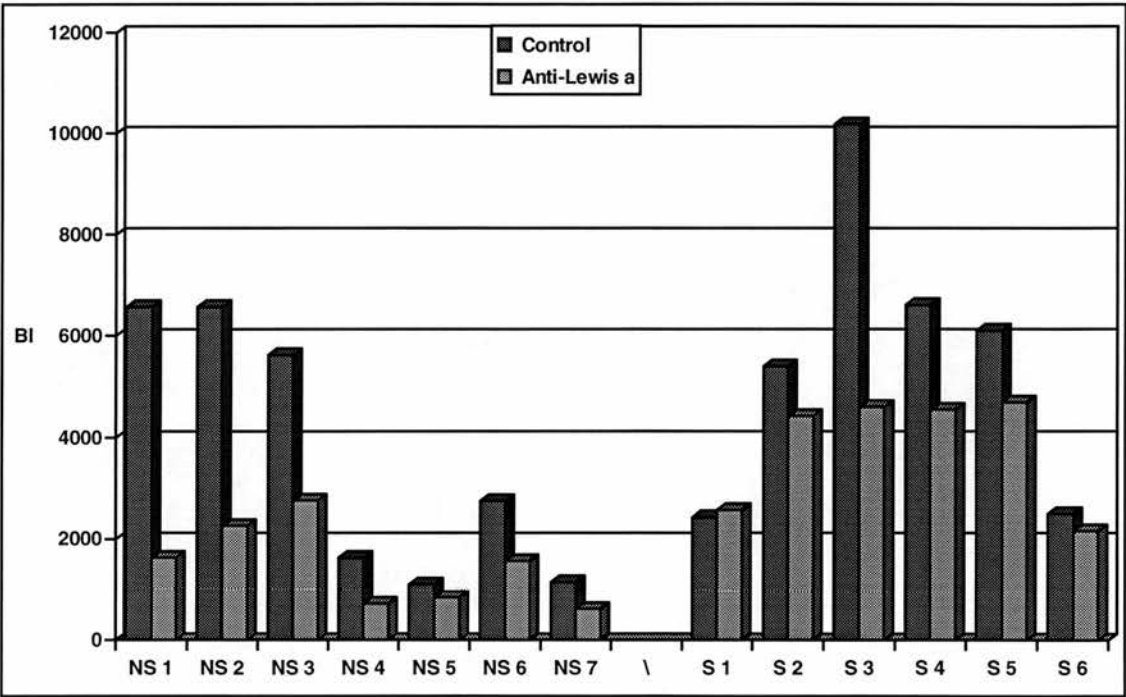


Figure 4.11 Effect of incubation of anti-precursor type 1 with BEC from NS and S donors on binding of *S. aureus* NCTC 10655.



**Figure 4.12** The effect of incubation of anti-Lewis<sup>x</sup> with BEC from 6 donors on binding of *S. aureus* NCTC 10655.



**Figure 4.13** Effect of incubation of anti-Lewis<sup>a</sup> with BEC from NS and S donors on binding of *S. aureus* NCTC 8532.

### **4.3.9 Binding of bacteria with reference to detection of Le<sup>a</sup> antigen, precursor antigen or Le<sup>x</sup>**

Binding of *S. aureus* NCTC 10655 correlated with the amount of anti-Le<sup>a</sup> antibody detected on the BEC of 8 secretors and 7 non-secretors ( $t = 5.03$ , d.f. = 12,  $P < 0.001$ ) (Table 4.5). A similar pattern was found with NCTC 8532 ( $t = 4.24$ , d.f. = 12,  $P < 0.001$ ) (Table 4.6). There was no significant correlation between the amount of anti-precursor type 1 detected on BEC and binding of NCTC 10655 or NCTC 8532 (Table 4.7). There was no correlation between the amount of Le<sup>x</sup> detected on BEC and binding of NCTC 10655 (Table 4.8).

## **4.4 Discussion**

The objectives of this chapter were to assess the level of expression of Le<sup>a</sup> on BEC of secretors and non-secretors and to determine if the *S. aureus* strains examined in the binding assays have adhesins that recognise Le<sup>a</sup>.

ELISA readings in the assay for Le<sup>a</sup> with saliva of non-secretors were 4-5 times greater than those for secretors, but there was considerable variability in the OD readings among the secretors. There were generally low values for Le<sup>b</sup> among the non-secretors. The OD readings for Le<sup>b</sup> were variable with the saliva of secretors. Very low readings for both Le<sup>a</sup> and Le<sup>b</sup> were obtained with saliva from donors whose erythrocytes were not agglutinated by anti-Le<sup>a</sup> or anti-Le<sup>b</sup>. These results gave expected values as non-secretors will form mainly Le<sup>a</sup> from precursor type 1 and Le<sup>b</sup> can not be formed in their secretions.

**Table 4.5 Correlation between binding of anti-Le<sup>a</sup> and binding of *S. aureus* NCTC 10655 to cells of secretors and non-secretor donors.**

| <u>Donors</u>       |   | <u>Anti-Le<sup>a</sup> (BI)</u> | <u>NCTC 10655 (BI)</u> |
|---------------------|---|---------------------------------|------------------------|
| <u>Secretor</u>     | 1 | 253                             | 2079                   |
|                     | 2 | 586                             | 1911                   |
|                     | 3 | 602                             | 3082                   |
|                     | 4 | 663                             | 2256                   |
|                     | 5 | 796                             | 3814                   |
|                     | 6 | 805                             | 2223                   |
|                     | 7 | 939                             | 2694                   |
|                     | 8 | 1049                            | 4152                   |
| <u>Non-secretor</u> | 1 | 445                             | 718                    |
|                     | 2 | 998                             | 1138                   |
|                     | 3 | 1143                            | 1210                   |
|                     | 4 | 1598                            | 3508                   |
|                     | 5 | 1806                            | 2532                   |
|                     | 6 | 1980                            | 4031                   |
|                     | 7 | 2490                            | 2721                   |

**Table 4.6 Correlation of binding of anti-Le<sup>a</sup> and binding of *S. aureus* NCTC 8532 to cells of secretors and non-secretor donors.**

| <u>Donors</u>       |   | <u>Anti-Le<sup>a</sup>(BI)</u> | <u>NCTC 8532(BI)</u> |
|---------------------|---|--------------------------------|----------------------|
| <u>Secretor</u>     | 1 | 253                            | 2178                 |
|                     | 2 | 586                            | 2347                 |
|                     | 3 | 602                            | 2275                 |
|                     | 4 | 663                            | 1826                 |
|                     | 5 | 796                            | 3262                 |
|                     | 6 | 805                            | 3268                 |
|                     | 7 | 939                            | 2378                 |
|                     | 8 | 1049                           | 3262                 |
| <u>Non-secretor</u> | 1 | 445                            | 2718                 |
|                     | 2 | 998                            | 6648                 |
|                     | 3 | 1143                           | 5462                 |
|                     | 4 | 1598                           | 6780                 |
|                     | 5 | 1806                           | 6266                 |
|                     | 6 | 1980                           | 9984                 |
|                     | 7 | 2490                           | 8201                 |

**Table 4.7 Correlation of binding of anti-precursor type 1 and binding of *S. aureus* NCTC 10655 or NCTC 8532 to BEC of secretor and non-secretor donors.**

| <b>(BI)</b>                |                                |                          |                         |
|----------------------------|--------------------------------|--------------------------|-------------------------|
| <b><u>Donors</u></b>       | <b><u>Precursor type 1</u></b> | <b><u>NCTC 10655</u></b> | <b><u>NCTC 8532</u></b> |
| Le <sup>a</sup> -b-        | 147                            | 518                      | 528                     |
| <b><u>Secretor</u></b>     |                                |                          |                         |
| 1                          | 831                            | 2855                     | 4035                    |
| 2                          | 1036                           | 4283                     | 7801                    |
| 3                          | 1356                           | 1726                     | 4446                    |
| <b><u>Non-secretor</u></b> |                                |                          |                         |
| 1                          | 247                            | 1344                     | 7047                    |
| 2                          | 605                            | 6514                     | 7256                    |
| 3                          | 3046                           | 790                      | 2760                    |



**Table 4.8 Correlation of binding of anti-Le<sup>x</sup> to binding of *S. aureus* NCTC 10655 to BEC of non-secreter donors.**

| <u>(BI)</u>   |                            |                   |
|---------------|----------------------------|-------------------|
| <u>Donors</u> | <u>Anti-Le<sup>x</sup></u> | <u>NCTC 10655</u> |
| 1             | 59                         | 5547              |
| 2             | 151                        | 2322              |
| 3             | 136                        | 2070              |
| 4             | 194                        | 5049              |
| 5             | 356                        | 2512              |
| 6             | 604                        | 3564              |

By flow cytometry, BEC from non-secretors generally bound more anti-Le<sup>a</sup> than those from secretors. Secretors bound highly variable amounts of anti-Le<sup>a</sup>. BEC from donors who were Le<sup>a-b-</sup> by haemagglutination had low BI for anti-Le<sup>a</sup>. This could be due to anti-Le<sup>a</sup> cross-reacting with precursor type 1 [Good *et al.*, 1992] or these individuals had low levels of Le<sup>a</sup> not detectable by haemagglutination. The BI for anti-Le<sup>b</sup> bound to BEC for the secretors were higher than those for non-secretors. The values of anti-Le<sup>b</sup> for the non-secretors indicate that anti-Le<sup>b</sup> might be cross reactive with Le<sup>a</sup> as non-secretors do not express Le<sup>b</sup> in their saliva or on their BEC. Anti-Le<sup>b</sup> was not included in further experiments for flow cytometry.

The results obtained with flow cytometry agreed with those obtained by haemagglutination and ELISA methods. Individuals whose erythrocytes were agglutinated strongly by anti-Le<sup>a</sup> had high OD for anti-Le<sup>a</sup> with their saliva in the ELISA and high BI for anti-Le<sup>a</sup> bound to their BEC measured by flow cytometry. Individuals whose erythrocytes were agglutinated by anti-Le<sup>b</sup> had variable amounts of Le<sup>a</sup> in their saliva and on their BEC.

There was less cross-reactivity with anti-Le<sup>b</sup> in the ELISA than in the flow cytometry studies. The cross reactivity of anti-Le<sup>b</sup> was also pointed out by other workers [Good *et al.*, 1992].

Precursor type 1 was not detected on BEC of every donor and binding of the antibody did not correlate with the secretor status of the donors or the BI for anti-Le<sup>a</sup>. This structure was predicted to be the main epitope of Lewis negative ABH non-secretor individuals [Le Pendu, 1982]. There was no Lewis negative ABH non-secretor individual among the donors tested for the study.

Anti-Le<sup>x</sup> bound to BEC from Le<sup>a+</sup> and Le<sup>a-b-</sup> donors. It is most likely that the gene for Le<sup>x</sup> is different from that for Le<sup>a</sup> as BI values for both antibodies did not correlate.

The Le<sup>x</sup> antigen (CD 15) results from the transfer of the  $\alpha$  1-3 fucose residue to the  $\beta$  GlcNAc of the type 2 precursor chain (Oriol *et al.*, 1986). This antigen is also widely distributed in several cell types and secretions (Gooi *et al.*, 1993).

BEC from Le<sup>a-b-</sup> donors absorbed large amounts of molecules containing Le<sup>a</sup> determinants when they were incubated with non-secretor saliva. This is in agreement with other studies on absorption of Le antigens from plasma by Le<sup>a-b-</sup> red blood cells (Marcus and Cass, 1969). BEC from non-secretors absorbed less Le<sup>a</sup> from non-secretor saliva than the Le<sup>a-b-</sup> cells. This indicates that the Le<sup>a</sup> was near saturation levels on the BEC of non-secretors and this made it difficult to adsorb more molecules as compared with BEC of Lewis negative donors. Some sort of exchange might also occur especially in the cells with precursor type 1. There was much less enhancement of BI for cells from either the Le<sup>a-b-</sup> donor or non-secretor donor incubated with saliva from Le<sup>a-b-</sup> and these results support what is expected as saliva from Le<sup>a-b-</sup> donors do not express Le<sup>a</sup> in order to be uptaken by the cells; in contrast, they might add some precursor type 1 molecules to the cells.

Attachment of strain NCTC 10655 which bound in greater numbers to non-secretor cells was significantly inhibited by treatment of the host cells with monoclonal anti-Le<sup>a</sup> antibody, monoclonal anti-precursor type-1 and anti-Le<sup>x</sup> antibody. Anti-precursor type-1 antibody was examined because it has been suggested that bacteria might bind to the precursor portion of the ABO or Lewis blood group antigens; and glycosylation of the precursor to H or Lewis antigens decreased the accessibility of the binding site (Rosenstein *et al.*, 1988). Anti-Le<sup>x</sup> antibody was included in the study as Le<sup>x</sup> is the type 2 isomer of Le<sup>a</sup>. Both Le<sup>a</sup> and Le<sup>x</sup> are oligosaccharides having the same sugar composition but differ stereochemically.

There was a significant correlation between binding of NCTC 10655 and binding of anti-Le<sup>a</sup> antibody to the host cell (Table 4.4), but there was no correlation between

binding of this strain with the amount of either anti-precursor or anti-Le<sup>x</sup> antibodies detected on the cells. As the anti-precursor monoclonal was prepared by immunisation with Le<sup>a</sup> antigen, the inhibition of bacterial binding observed following pre-treatment of cells with anti-precursor might be due to cross-reactivity with Le<sup>a</sup> although none was indicated by the manufacturer. The inhibition of bacterial binding following pre-treatment of cells with anti-Le<sup>x</sup> might be due to the adhesins recognising a carbohydrate domain common to both Le<sup>a</sup> and Le<sup>x</sup> antigens.

Although NCTC 8532 did not bind in greater numbers to non-secretor cells compared with secretor cells (Fig 3.10), its binding was inhibited by treatment of the epithelial cells with anti-Le<sup>a</sup> (Fig 4.13) and there was a significant correlation between its binding to the donor cells and the amount of anti-Le<sup>a</sup> antibody bound by the cells. Some secretors bind as much anti-Le<sup>a</sup> as most non-secretors. For the experiments comparing the binding of staphylococci to cells from secretors and non-secretors, the donors were matched only according to, sex, age and ABO blood group; and the same pairs of secretor / non-secretor donor were not available for each experiment. At that time there was no information on the amount of anti-Le<sup>a</sup> bound by the cells of individual donors.

Further evidence that both NCTC 10655 and NCTC 8532 have adhesins that bind Le<sup>a</sup> was obtained with co-agglutination studies using anti-idiotypic antibodies produced against monoclonal anti-Le<sup>a</sup>, the Fab portions of which resemble the epitope bound by the monoclonal anti-Le<sup>a</sup>. All the staphylococcal strains tested were agglutinated : NCTC 8532, NCTC 10652, NCTC 10654, NCTC 10655, NCTC 11965 and 41206 [Essery *et al.*, 1994b].

The results of this chapter indicate that:

1. The adhesin that bind Le<sup>a</sup> is present on all strains tested.

2. Binding of NCTC 10655 and NCTC 8532 was correlated with the binding of anti-Le<sup>a</sup>.

3. Differences in binding of staphylococcal strains observed in chapter 3 are not due to absence of adhesins that bind anti-Le<sup>a</sup>.

4. Expression of Le<sup>a</sup> represented in binding of anti-Le<sup>a</sup> on secretors is highly variable.

Isolation of the staphylococcal adhesins that recognise Le<sup>a</sup> is the subject of the next chapter.

## Chapter 5

# Isolation of an adhesin from *Staphylococcus aureus* that binds Lewis<sup>a</sup> blood group antigen

### 5.1 Introduction

Epidemiological studies have found that individuals who are non-secretors of the ABO blood group antigens are over-represented among patients with some bacterial diseases and among carriers of some potentially pathogenic bacteria. The Le<sup>a</sup> antigen, present in greater quantities on epithelial cells of non-secretors, might be a receptor for some adhesins on strains of these bacterial pathogens. If so, individuals who express significant amounts of Le<sup>a</sup> might be more readily or more densely colonized by these organisms [Blackwell, 1989b].

The results in chapter 4 indicated that binding of both toxigenic and non-toxigenic strains of *S. aureus* to epithelial cells was correlated with the amount of Le<sup>a</sup> antigen detected on the cells; and, monoclonal antibody to this antigen can block attachment of these bacteria. These results suggest that some surface-exposed proteins on *S. aureus* strains might be adhesins that use Le<sup>a</sup> as a receptor.

Adhesion of staphylococci to various tissues involves non-specific, hydrophobic interactions, as well as biospecific interaction, via binding proteins [Vercellotti *et al.*, 1984]. *S. aureus* has a large number of agglutinogens; however, identification of specific adhesins that mediate binding to eukaryotic cells is limited. In this chapter an attempt was made to isolate the surface component of *S. aureus* that binds Le<sup>a</sup> antigen and to use it to reduce attachment of the bacteria to epithelial cells.

## **5.2 Materials and methods**

### **5.2.1 Bacterial strains**

Lyophilised *S. aureus* strains NCTC 10655, NCTC 10652 and NCTC 8532 were cultured on nutrient agar for 24 h at 37°C. For each strain, growth from 20 plates was collected; this yielded (2g) of bacterial pellet.

### **5.2.2 Cell wall isolation**

A modification of methods described previously [Cheung *et al.*, 1988; Sharp and Poxton, 1988] was used. Bacteria were suspended in washing buffer (0.05M Tris HCl, 0.025M MgCl<sub>2</sub> and 0.15M NaCl, pH 7.6) and centrifuged for 20 min at 2000 g at 4°C. The bacterial pellet (2g) was suspended in 15 ml of freshly prepared washing buffer containing DNase-1 (50 ug) (BDH, 39012) and RNase (50 ug), (Calbiochem, lot 902237). The pre-cooled (4°C) cell suspension was passed twice through a French Pressure Cell (American Instrument Co., Silver Spring, Md. USA)  $p_a = 6000\text{--}7000 \text{ lb/in}^2$  then centrifuged at 2000 g for 20 min at 4°C to remove intact bacteria. The supernatant was then centrifuged at 20,000 g for 30 min to recover the cell wall material. The resulting pellet was washed 3 times in ice cold distilled water by centrifugation at 30,000 g for 15 min at 4°C.

### **5.2.3 Extraction of cell wall protein**

The pellet from the crude cell wall preparation was resuspended at room temperature in 5 ml of 2% (v/v) Triton X-100 (Sigma T-6878) in water for 30 min and centrifuged at

20,000 g for 20 min. After two such extractions, the pellet was dissolved in 5 ml of a freshly prepared solution containing 50 µg/ml lysostaphin (Sigma L-7386) in 0.05 M Tris/HCl with 0.145 M NaCl (pH 7.6) and incubated at 37°C for 2h with continuous shaking. After centrifugation at 45,000 g for 20 min at 4°C, the supernatant was freeze dried, reconstituted in distilled water and stored at -20°C.

## **5.2.4 Purification of adhesins by affinity adsorption**

Synsorb affinity adsorbent (100 mg) (ChemBiomed Ltd., Edmonton, Alberta, Canada; lot Asi-137) with Le<sup>a</sup> covalently linked to the silica matrix was swollen in a Falcon tube in 2 ml of PBS (pH 7.2) for 2 h at RT with continuous rotation. The beads were centrifuged at 50 g for 5 min and the supernatant removed. The protein extract (0.17 mg in 1 ml) from strain NCTC 10655 was added to the beads, rotated overnight at 4°C and centrifuged at 50 g for 5 min. The supernatant containing unbound material was collected. The beads were washed twice with 2 ml of PBS and the washings added to the unbound material. The bound material was eluted from the beads with 1 ml of 2% ammonia in saline for 20 min with continuous rotation at room temperature. The supernatant was recovered following centrifugation at 50 g for 5 min. The beads were finally washed with 2 ml of PBS and the eluate added to the previous supernatant. The original sample and all extracts were freeze dried, reconstituted in distilled water and dialysed overnight against Tris / HCl (0.01 M, pH 7.4) at 4°C.



### 5.2.5 Polyacrylamide gel electrophoresis (PAGE)

The buffer system of Laemmli [1970] with 10% acrylamide in a Biometra-Minigel (Biometra, Wagen-Stieg, Gottingen, Germany) was used. Protein content was determined by the method of Bradford [1976] using Coomassie brilliant blue with bovin serum albumin as standard. Samples (20  $\mu$ l) containing 8  $\mu$ g protein were applied to each lane of the gel for 1-2 h, at 60 volts for the stacking gel (5% acrylamide) and 150 volts through the resolving gel (10% acrylamide) to complete the run and stained with Coomassie blue. Molecular weight markers (Sigma) in the range of 29-205 kDa were run in parallel.

### 5.2.6 Inhibition assay

The affinity purified extract and the unbound material of NCTC 10655 prepared as above (5.2.4) were made up to a protein concentration of 15  $\mu$ g/ml. A volume (100  $\mu$ l) of each was added to 100  $\mu$ l suspensions of BEC ( $2.5 \times 10^5$ /ml) taken from 3 healthy individuals expressing Le<sup>a</sup>. BEC incubated with 100  $\mu$ l of PBS were included as untreated controls. After incubation for 1 h at room temperature, the cells were washed twice with PBS by centrifugation at 300 g for 10 min. *S. aureus* NCTC 10655 (100  $\mu$ l) labelled with FITC by the method described in chapter 3 were added to the BEC at a ratio of 640 bacteria/cell. After 30 min at 37°C with continuous shaking, the cells were washed twice with PBS, fixed with 1% paraformaldehyde and analysed by flow cytometry as previously described in Chapter 3. The binding index (BI) for each sample was calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel of the positive population. The percent inhibition was calculated by the formula:

$$100 - [(BI \text{ test} / BI \text{ control}) \times 100].$$

## 5.3 Results

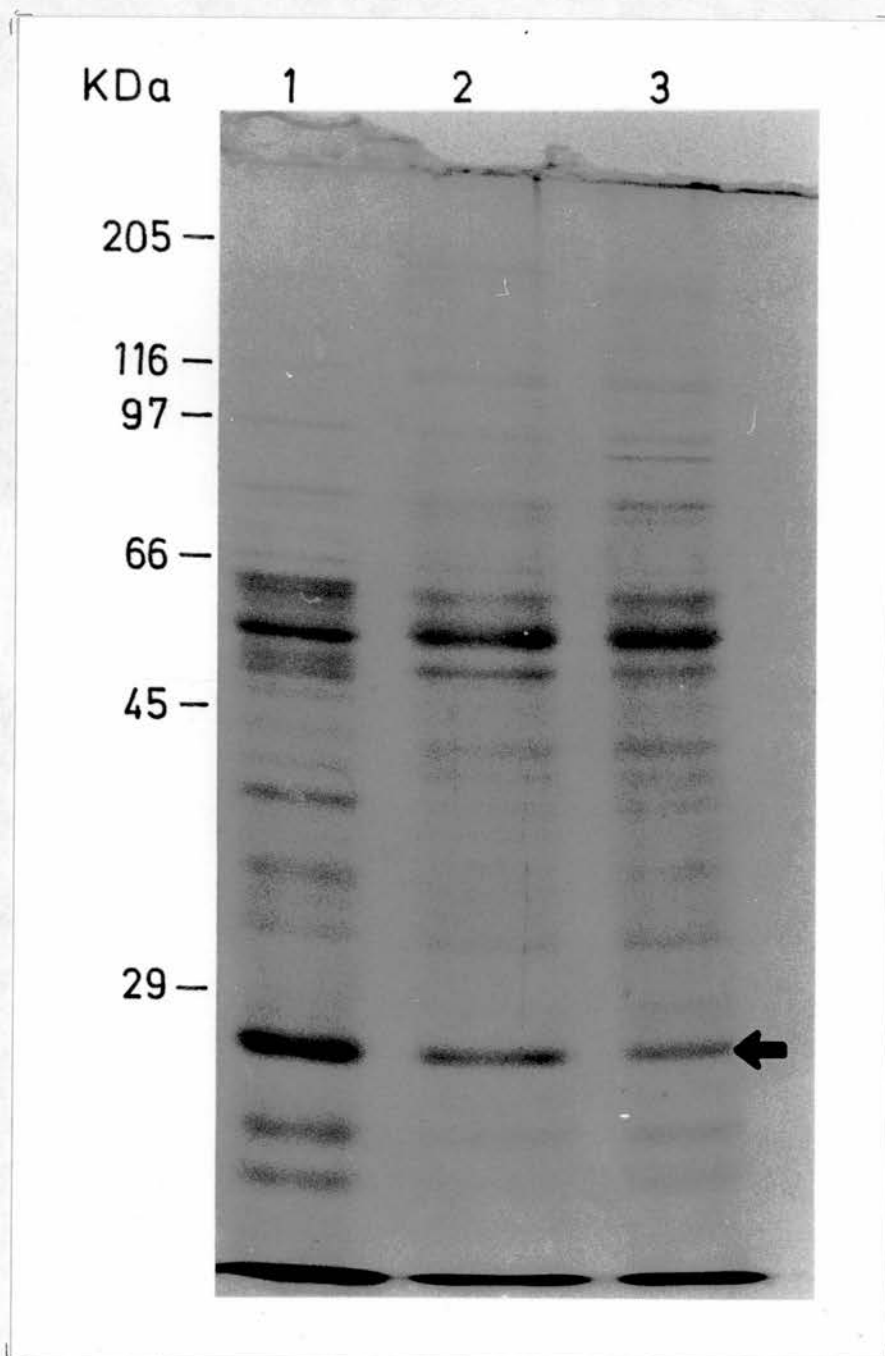
### 5.3.1 Cell wall preparation

The protein profiles by PAGE of the material solubilised by lysostaphin from strains NCTC 10655, NCTC 10652 and NCTC 8532 are shown in Fig 5.1. The band at 28 kDa is the lysostaphin. All three strains gave almost identical patterns.

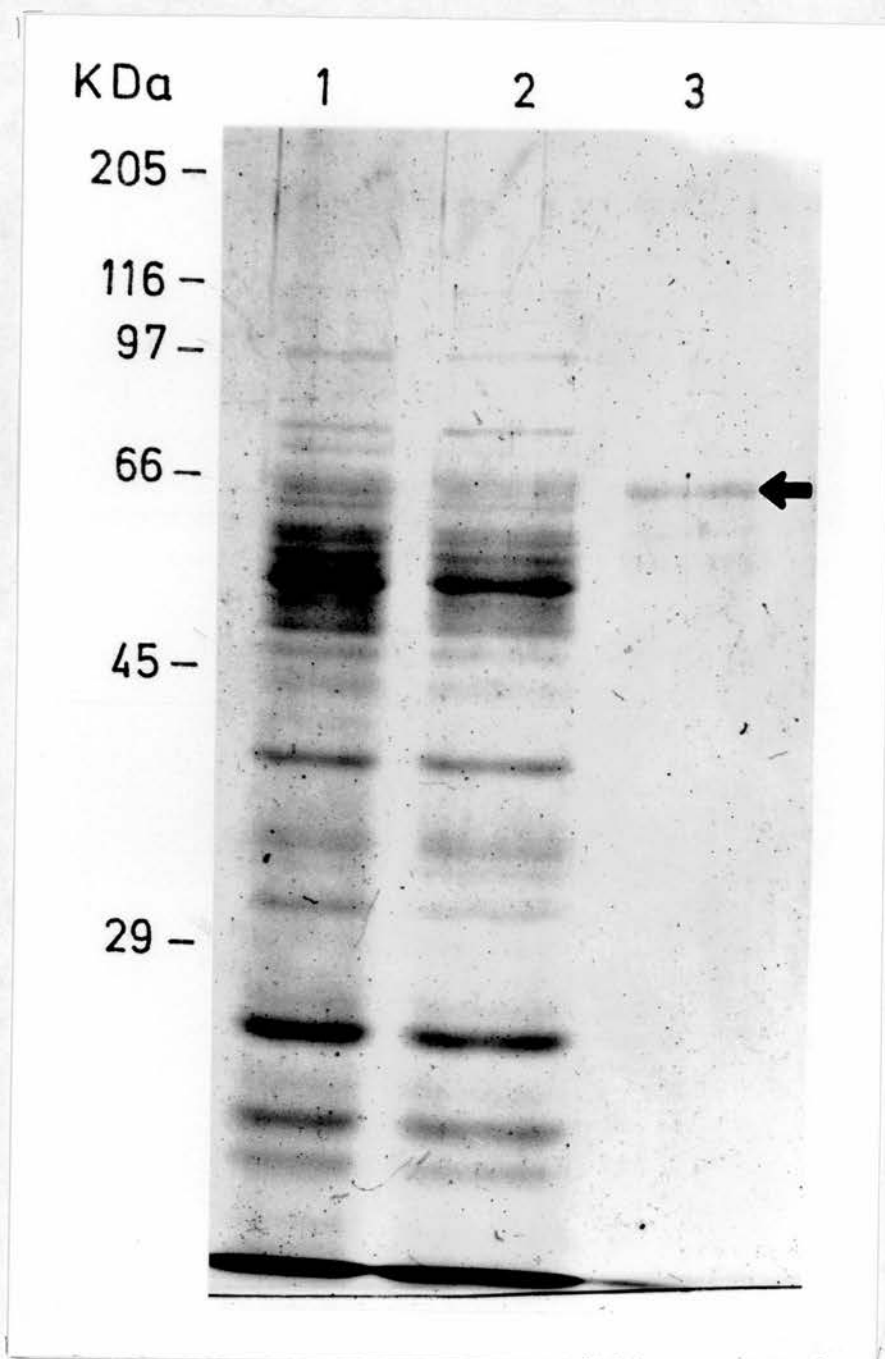
Fig 5.2 shows the peptide bands of strain NCTC 10655 before and after adsorption with Synsorb Le<sup>a</sup>. The bands for the unbound material (track 2) and the original cell wall material (track 1) show similar patterns of multiple bands while the eluate (track 3) has a major band of approximately 67 kDa. The protein concentration of the original cell wall extract was 170 µg/ml and that of the unbound material and Synsorb eluate were 150 µg/ml and 15 µg/ml respectively.

### 5.3.2 Inhibition studies

The ability of the protein preparations to inhibit binding of *S. aureus* NCTC 10655 to BEC was assessed by flow cytometry. Three experiments were performed. In each experiment, the percentage inhibition was greater for the protein eluted from Synsorb Le<sup>a</sup> (Table 5.1). The eluate from a control preparation obtained by passing lysostaphin solution through the Synsorb beads showed no inhibition of bacterial binding to buccal epithelial cells (data not shown).



**Fig 5.1** Coomassie brilliant blue-stained SDS-PAGE (10%) of proteins (8  $\mu$ g) extracted from *S. aureus* NCTC 10655 (lane 1), NCTC 10652 (lane 2), NCTC 8532 (lane 3).



**Fig 5.2** Commassie brilliant blue-stained SDS-Page (10%) of cell wall extracts of *S. aureus* NCTC 10655 (lane 1), unbound material (lane 2), and Synsorb-Lewis<sup>a</sup> eluate (lane 3).

**Table 5.1 Inhibition of binding of *S. aureus* NCTC 10655 by pre-treatment of BEC with protein preparation (15 µg/ml) eluted from Synsorb-Le<sup>a</sup> or the unbound material.**

| <u>% inhibition</u> |                      |                        |                                      |
|---------------------|----------------------|------------------------|--------------------------------------|
| <u>Experiment</u>   | <u>Whole extract</u> | <u>Unbound protein</u> | <u>Synsorb-Le<sup>a</sup> eluate</u> |
| 1                   | 44.8                 | 25.5                   | 54.8                                 |
| 2                   | ND*                  | 6.5                    | 24                                   |
| 3                   | ND*                  | 13.3                   | 25.7                                 |

(\*ND = not done).

## 5.4 Discussion

The Synsorb-Le<sup>a</sup> affinity purification isolated a major polypeptide of 67 kDa from outer membranes of *S. aureus*. The cell wall preparations were capable of inhibiting attachment of strain NCTC 10655 to buccal epithelial cells; the inhibitory activity of the eluate from Synsorb Le<sup>a</sup> was greater than that of the unbound material. This supports the results in chapter 4 which found that binding of *S. aureus* strains to BEC was significantly correlated with the amount of Le<sup>a</sup> on the cells and binding of both NCTC 10655 and NCTC 8532 to these cells could be significantly reduced by pre-incubation of the cells with monoclonal anti-Le<sup>a</sup>.

Complete inhibition of binding was not predicted because there are many receptors for *S. aureus* on epithelial cells [Sandford, 1986]. *S. aureus* cell wall components, lipoteichoic acid [Carruthers and Kabat, 1983], teichoic acids [Aly *et al.*, 1980] and protein A [Cole and Silverberg, 1986; Wyatt, 1990] have been implicated in adherence.

Relatively little is known about cell-wall proteins of *S. aureus* other than protein A. Another cell wall protein previously identified is a fibronectin-binding protein of 210 kDa [Ciborowski *et al.*, 1992]. The fibronectin receptor is expressed in greater quantities on invasive *S. aureus* isolates than non-invasive isolates; and it has been implicated in adherence of *S. aureus* to squamous epithelium [Aly, 1987]. Fibronectin binding is not regarded as an important mechanism of adhesion to a host tissue where a large number of both protein and carbohydrate components could conceivably be recognised by microbial adhesins. The molecular mass of the Le<sup>a</sup> adhesin 67 kDa differs from those reported for adhesins binding fibrinogen (19 kDa, 60 kDa and 87 kDa) [Boden and Flock, 1992] or fibronectin (210 kDa) [Ciborowski *et al.*, 1992].

Surface protein antigens are probably important in eliciting host immune responses to *S. aureus* infection [Cheung, 1988]. The adhesin isolated from this study could be useful in defining markers of epidemiological importance and antibodies to this adhesin in secretions and milk need to be explored with reference to colonisation of infants by toxigenic bacteria.



## Chapter 6

### The role of *Bordetella pertussis* in SIDS

#### 6.1 Introduction

*B. pertussis* colonises the respiratory tract and causes infant death from whooping cough (pertussis). It has been suggested that some cases of SIDS are due to asymptomatic infection with *B. pertussis* [Nichol and Gardner, 1988], or alternatively, caused by immunization for diphtheria, pertussis and tetanus (DPT) [Torch, 1982 and Baraff *et al.*, 1983]. Epidemiological studies did not confirm the hypothesis regarding immunization; the data indicated that the unimmunised infants were at greater risk of SIDS [Emery, 1981; Hoffman *et al.*, 1987 and Walker *et al.*, 1987].

There is no evidence of systematic surveys for these bacteria in SIDS infants; and the bacteria are difficult to culture even from children with symptomatic whooping cough [Davis *et al.*, 1990]. Consequently, there are no reports that *B. pertussis* has been isolated from SIDS infants.

This micro-organism is well endowed with potential adhesins: filamentous haemagglutinin (FHA); pertussis toxin (PT); and fimbriae. Both FHA and PT are exposed on the surface of *B. pertussis*, but their relative contributions to colonization of epithelial surfaces are not clear [Tuomanen and Weiss, 1985]. *B. pertussis* can bind to monocytes through its toxin; and the toxin has been demonstrated to bind Le<sup>a</sup> and Le<sup>x</sup> antigens on these cells [van t'Wout *et al.*, 1992]; however, there were no reports on the role of these antigens in colonization of epithelial surfaces by *B. pertussis*.



In addition to the campaign to place infants on their backs to sleep, there have been other changes in infant health care that might have contributed to reduction in SIDS observed in Scotland since 1990. As well as initiation of immunization for DPT at the earlier age of 2 months, there has been a return of confidence in pertussis immunization reflected in the steady increase in vaccination uptake from the late 1980's [Preston, 1993]. If some cases of SIDS were due to asymptomatic pertussis, earlier immunization might help prevent a portion of these death. This might be reflected in a shift in the age distribution of SIDS cases with an increase in the younger age group.

The aim of this chapter was to test two hypothesis: first that colonization of young infants by *B. pertussis* might be enhanced by the bacteria binding to Le<sup>a</sup> and Le<sup>x</sup> on epithelial cells; second, there would be an increase in the proportion of unimmunized infants ( $\leq 2$  months of age) among SIDS cases following initiation of DPT immunization at 2 months instead of 3 months.

## **6.2 Materials and methods**

### **6.2.1 Preparation of bacteria**

Two strains of *B. pertussis* were kindly provided by Dr. N.W. Preston, Department of Microbiology, Manchester University: 8002 (fimbriate, type 1,2); 250815 (non-fimbriate, type 1,3). Both strains were grown on charcoal blood agar plates and incubated for 6 days at 37°C. For storage, colonies were emulsified in Microbank beads and kept at -20°C. A fresh bead was used to inoculate plates for each set of experiments.

## **6.2.2 Total bacterial count**

Total numbers of *B. pertussis* were determined by the optical density method described in 2.8.3.

## **6.2.3 Binding of bacteria to BEC**

Binding of bacteria labelled with FITC to BEC of healthy donors was assessed by the flow cytometry method as described in 3.2.3.4.

## **6.2.4 Detection of Le<sup>a</sup> and Le<sup>x</sup> on BEC**

The flow cytometry method described in chapter 3 was used to detect Le<sup>a</sup> and Le<sup>x</sup> antigens on BEC.

## **6.2.5 Inhibition of bacterial binding**

The method described in 4.2.5 was used in these experiments. BEC were treated with PBS, monoclonal anti-Le<sup>a</sup> (1/2) or anti-Le<sup>x</sup> (1/10). The attachment assay was then performed with *B. pertussis* (500 bacteria : cell). The results were analysed by flow cytometry.

## **6.2.6 Statistical methods**

Statistical analyses were carried out on the logarithms of the binding indices. Differences between groups were tested by paired or unpaired *t*-tests as appropriate, and confidence limits for the mean values in one group were expressed as percentage of those in the other by taking antilogarithms.

Statistics on the number of cases of SIDS was obtained from the Scottish Cot Death Trust national survey. Ages of SIDS cases in the Lothian, Borders and Fife area from 1988 to 1992 were obtained from autopsy reports. The change in the proportion of cases  $\leq 2$  months of age over time was tested using a chi-squared test for trend.

## **6.3 Results**

### **6.3.1 Correlation of total bacterial count with OD**

The linear correlation between readings of OD with total bacterial count for both strains of *B. pertussis* tested is shown in Figure 6.1.

### **6.3.2 Optimal time for bacterial binding**

At a concentrations of 500 bacteria / cell of FITC-labelled *B. pertussis* were incubated with BEC at 45, 120 and 180 min. Attachment was adequate at 120 min (Figure 6.2) and further binding assays were performed at this incubation time.

### **6.3.3 Bacterial binding dose response**

Binding of *B. pertussis* strain 8002 and strain 250815 to BEC were performed with five different ratios of bacteria per cell : 63, 125, 250, 500 or 1000 : 1. Figure 6.3 shows the dose response effect. Bacterial binding was sufficient at 500 bacteria per cell and this ratio was used for further experiments.

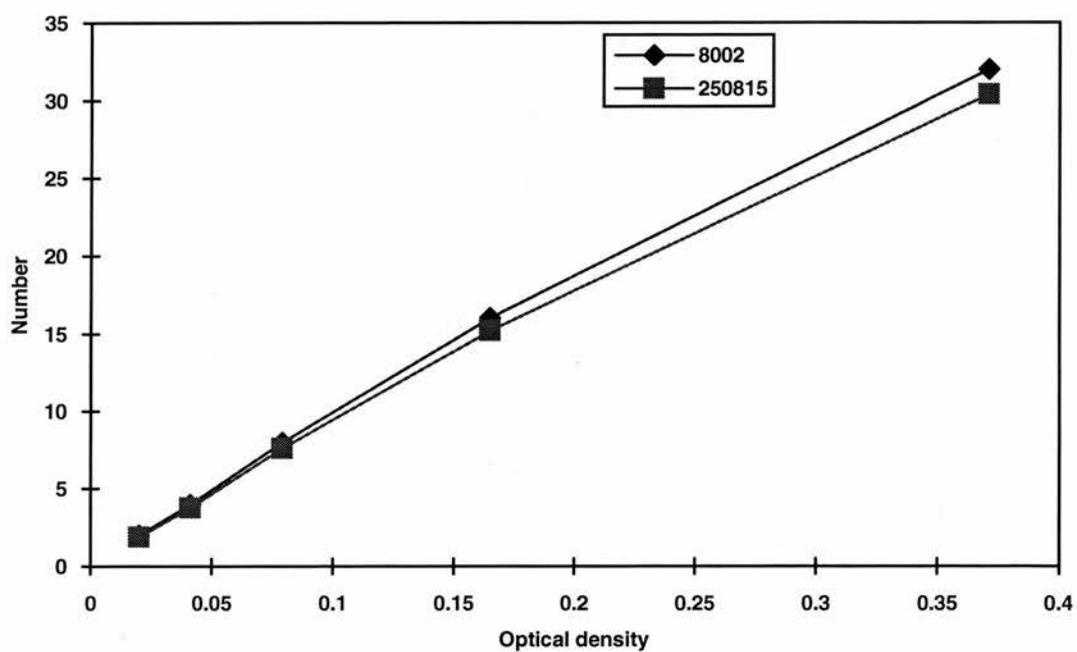
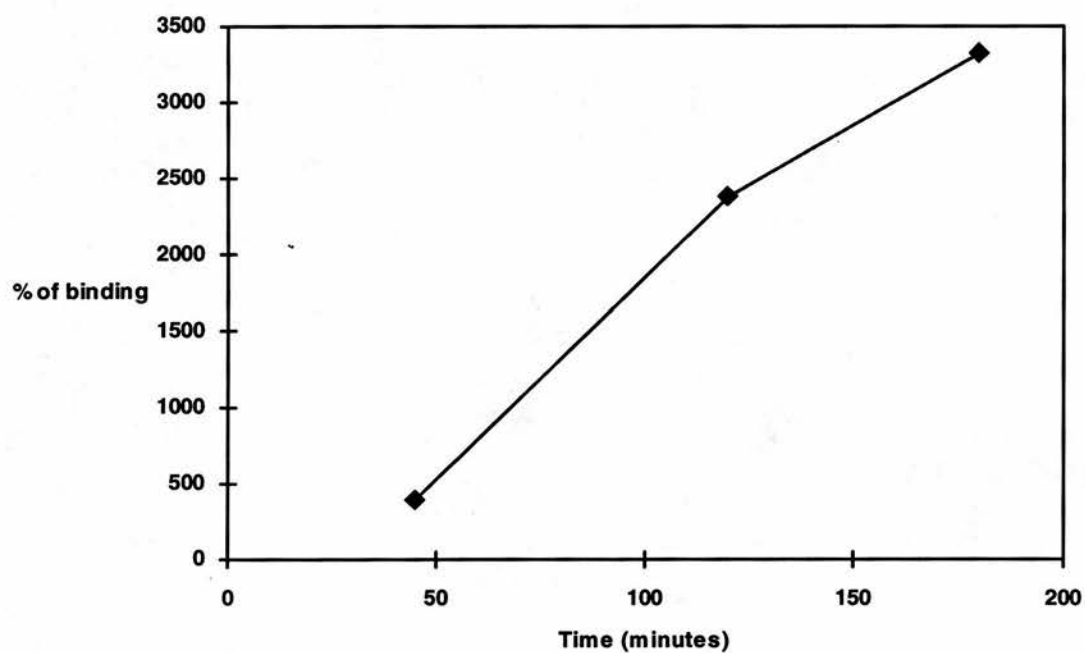
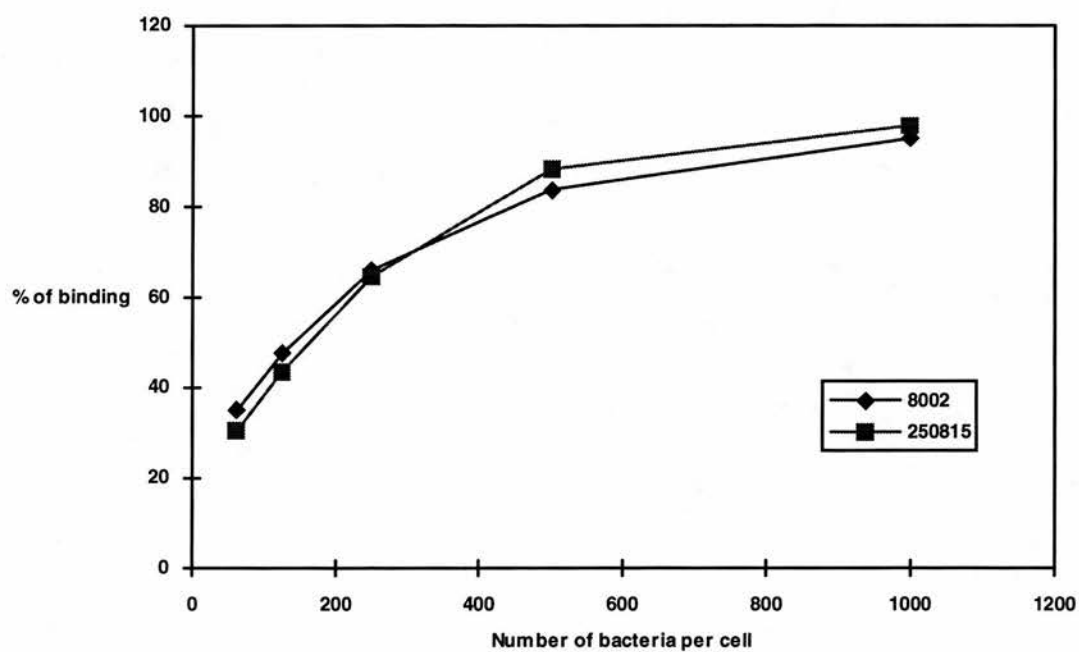


Figure 6.1 Total bacterial count (number  $\times 10^{-9}$ ) per ml determined by microscopy versus optical density of *B. pertussis* strains 8002 and 250815.



**Figure 6.2** Time course for binding of *B. pertussis* strain 8002 to BEC.



**Figure 6.3 Dose response for *B. pertussis* strain 8002 and strain 250815 binding to BEC.**

### 6.3.4 Inhibition of bacterial binding

Figure 6.4 compares binding of *B. pertussis* strain 8002 to epithelial cells treated with PBS, anti-Le<sup>a</sup> or anti-Le<sup>x</sup>. Binding of 8002 to cells treated with anti-Le<sup>a</sup> was significantly lower compared with the control treated with PBS ( $t = -2.93$ , CI 86-99%,  $p < 0.05$ ). Pretreatment of cells with anti-Le<sup>x</sup> also significantly reduced binding of 8002 ( $t = -4.32$ , CI 45-82%,  $p < 0.01$ ). Although there appeared to be less Le<sup>x</sup> antigen present on the cells, inhibition of bacterial binding was greater if the cells were pre-treated with anti-Le<sup>x</sup> than with Le<sup>a</sup> ( $P < 0.01$ , 95% CI 51-84%). Similar results were obtained with strain 250815 (Figure 6.5). Bacterial binding to cells treated with anti-Le<sup>a</sup> was significantly lower than the control (PBS). There was significant inhibition of binding with anti-Le<sup>a</sup> ( $t = -3.23$ , CI 71-94%,  $P < 0.05$ ) or with anti-Le<sup>x</sup> ( $t = -4.41$ , CI 64-86,  $P < 0.01$ ).

### 6.3.5 Age distribution of SIDS cases in Scotland

Although the number of SIDS cases in Scotland has decreased since 1990 (Figure 6.6), the proportion of infants in Lothian, Borders and Fife aged 2 months or less has increased from 16% in 1988 to 40% in 1992 ( $X^2 = 5.53$ ,  $P = 0.02$ ) (Table 6.1).

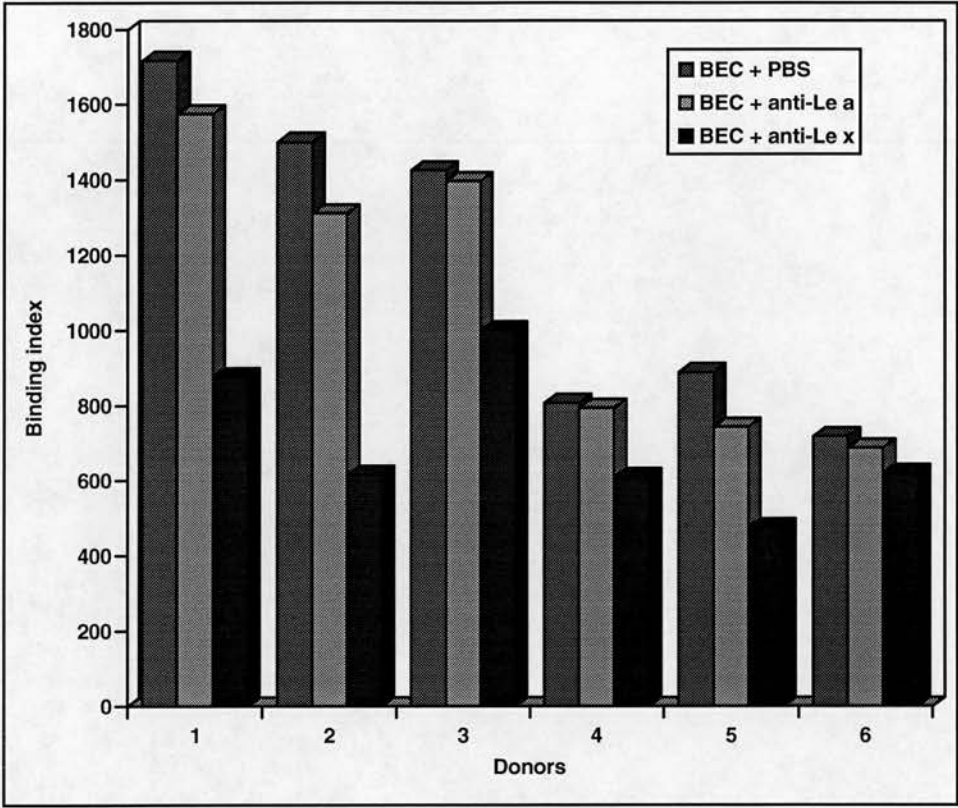


Figure 6.4 Inhibition of binding of *B. pertussis* strain 8002 by pre-treatment of BEC with monoclonal anti-Le<sup>a</sup> or anti-Le<sup>x</sup>.



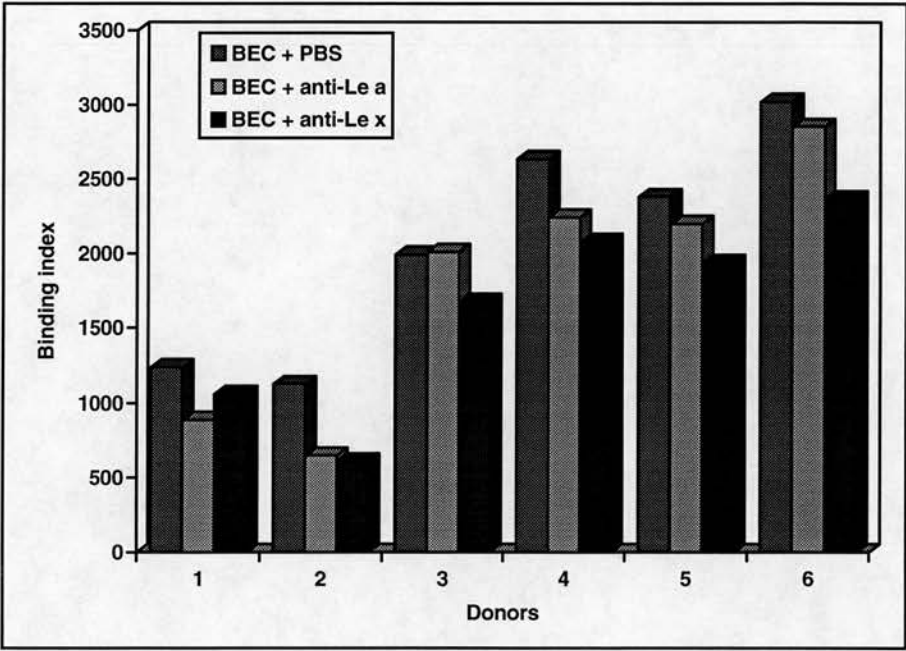
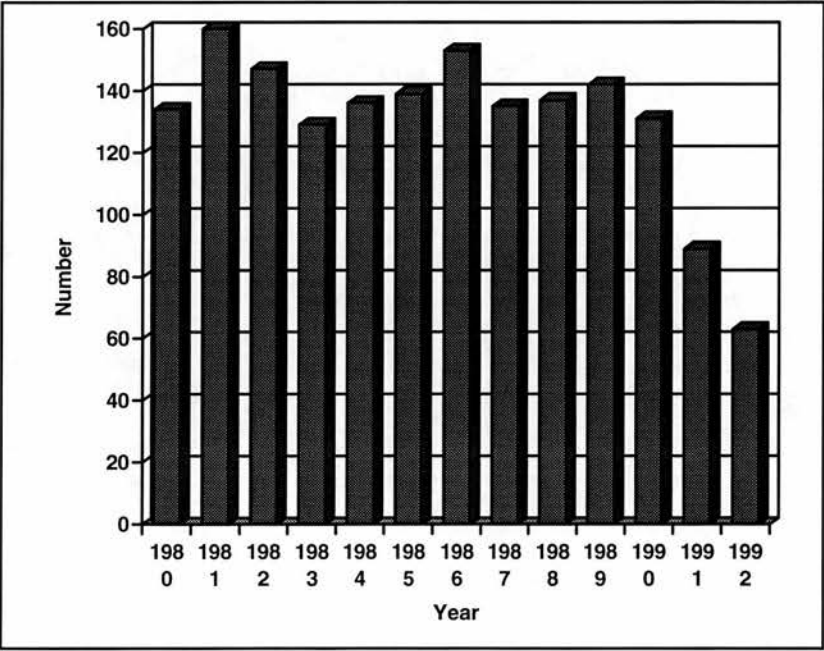


Figure 6.5 Inhibition of binding of *B. pertussis* strain 250815 by pre-treatment of BEC with monoclonal anti-Le<sup>a</sup> and anti-Le<sup>x</sup>.



**Figure 6.6 Cases of SIDS in Scotland 1980-1992**

**Table 6.1 Age distribution of SIDS cases in Lothian, Borders and Fife regions 1988-1992**

| <u>Year</u> | <u>Total</u> | <u>&lt;/2 months</u> | <u>&gt;2 months</u> |
|-------------|--------------|----------------------|---------------------|
|             |              | <u>(no%)</u>         |                     |
| 1988        | 25           | 4 (16)               | 22 (88)             |
| 1989        | 26           | 3 (11.5)             | 23 (88.5)           |
| 1990        | 31           | 9 (29)               | 22 (71)             |
| 1991        | 13           | 4 (31)               | 9 (69)              |
| 1992        | 10           | 4 (40)               | 6 (60)              |

## 6.4 Discussion

The results of chapter 3 and 4 identified factors which might enhance colonization of infants by toxigenic staphylococci during the 2-4 month age range in which most SIDS cases occur. A similar pattern was found in this study with *B. pertussis*. Binding of both strains of *B. pertussis* tested was significantly inhibited by pre-treatment of epithelial cells with either anti-Le<sup>a</sup> or anti-Le<sup>x</sup>. The pattern of inhibition was similar for both the strains tested.

As there is little maternal antibody against pertussis, young infants are very vulnerable to these bacteria. A proportion of SIDS in the unimmunized age range might be due to asymptomatic pertussis. Infants less than 6 months of age do not develop the typical paroxysms and the infection might not be diagnosed since the organisms are difficult to culture even from symptomatic patients. The cause of death in these infants could be hypoglycaemia. The predominant effect of pertussis toxin is stimulation of insulin from the pancreas [Davis *et al.*, 1990] and TNF  $\alpha$  produced in response to pertussis can also cause hypoglycaemia. In a Finnish study, two-third of the 57 SIDS infants were younger than 3 months; about half had a mild "virus-like" infection approximately one week before death and serum insulin levels were significantly lower for the SIDS infants compared with those who had died of other causes. Hyperplasia of the islet cells was observed in the SIDS infants [Hirvonen *et al.*, 1980].

The antibodies induced by the whole cells and the toxoid contained in the pertussis vaccine might also be cross reactive with shared epitopes on adhesins and toxins of other species. These crossreactive antibodies might contribute to decreased colonization by toxigenic bacteria or partially neutralise the effects of their toxins. The pertussis toxin acts as an adhesin and binds to Le<sup>a</sup> and Le<sup>x</sup> on human monocytes [van t'Wout *et al.*, 1992], the

finding that the staphylococcal enterotoxin B binds to Le<sup>a</sup> and Le<sup>x</sup> [Essery *et al.*, 1994a] is further evidence for this hypotheses; and immunological cross-reactivities between antibodies to staphylococcal pyrogenic toxins, pertussis toxin and whole cells of *B. pertussis* have been demonstrated by ELISA [Essery *et al.*, 1994c].

The increased uptake of pertussis vaccine during the past few years [Preston, 1993] and the change in immunization schedule might contribute in two ways to the observed decrease in SIDS noted since 1990. First, immunization would reduce those cases of SIDS suggested to be due to asymptomatic pertussis [Nichol and Gardner, 1988]. Studies with the ferret model found that viral infection greatly enhanced the effect of diphtheria toxin, if there is a parallel in human infants, DPT immunization might also reduce the risk of death due to these bacteria. Second, if immunization with pertussis toxoid produces antibodies cross-reactive with epitopes on adhesins or toxins of other bacterial species that bind Le<sup>a</sup> and / or Le<sup>x</sup>, this will induce immunity to pertussis and might also reduce the effects of toxigenic strains of staphylococci.

More detailed epidemiological studies of SIDS before and after October 1990 are needed to obtain additional evidence to support or refute this hypothesis. Studies to detect the presence of pertussis in the oropharynx of infants have been initiated and methods to detect the pertussis toxin in tissues are under development.

## Chapter 7

# Viral infections as predisposing factors for colonization by bacteria and their association with SIDS

### 7.1 Introduction

Although there is no direct evidence for viruses causing cot death [Fleming, 1992], they might be predisposing factors for colonization by toxigenic bacteria or exacerbate the effect of bacterial colonization [Jakeman *et al.*, 1991]. Much of the work on enhancement of bacterial colonization has been done on influenza virus and superinfection by staphylococci or pneumococci [Plotkowski *et al.*, 1984]. The involvement of viruses rests on clinical experience, epidemiological studies and from laboratory evidence that virus infection predispose people to some bacterial diseases by enhancing colonization by pathogenic bacteria [Ramirez-Ronda *et al.*, 1981 and McDonald *et al.*, 1987].

Respiratory syncytial virus (RSV) was taken as a model for this study because it is an important, ubiquitous respiratory pathogen causing infection in young infants during the winter months when SIDS is most common [Noah *et al.*, 1989]. In addition, this virus has been identified in SIDS infants [Ogra *et al.*, 1975; Williams *et al.*, 1984] and Ford *et al.*, [1990] reported a weak association with SIDS in a New Zealand study. Finally, cells infected with RSV have been shown to bind significantly more *Neisseria meningitidis* and type b *Haemophilus influenza* than uninfected cells [Raza *et al.*, 1993].

In this part of the study an attempt was made to determine if tissue culture cells infected with RSV bind greater numbers of *S. aureus* or *B. pertussis* than uninfected cells.



## **7.2 Materials and methods**

### **7.2.1 Bacteria**

Two strains of *S. aureus*, NCTC 10655 and NCTC 8532 and two strains of *B. pertussis*, a fimbriate strain 8002 and a non-fimbriate strain 250815, were used. The strains of *S. aureus* were grown on nutrient agar and fresh subcultures from an overnight growth at 37°C were used for each experiment. The strains of *B. pertussis* were grown on charcoal blood agar for 6 days at 37°C and a total bacterial count for each strain of both bacteria was performed by microscopy as described in 2.8.3.

### **7.2.2 Cells**

HEp-2 cells (Flow laboratories) were used for the binding assays. Cell growth medium consisted of Eagle 's minimal essential medium (Gibco) supplemented with 10% foetal calf serum (Gibco), NaHCO<sub>3</sub> (0.85 g/l), L-glutamine (2 mM), streptomycin (200 µg/ml) and penicillin (100 IU/ml). Maintenance medium contained all the constituents of cell growth medium but only 1% foetal calf serum. The cells used in the bacterial binding studies were suspended by applying 1 ml EDTA (0.05%) to monolayers in 25 cm<sup>2</sup> flask for 10 min at 37°C after the cells were suspended in growth medium or in maintenance medium.

### **7.2.3 Virus and virus infected cells**

Both the RSV infected and the uninfected HEp-2 cells were provided by Dr. M. W Raza, Department of Medical Microbiology.

One day old monolayer of HEp-2 cells in culture flasks (25 cm<sup>3</sup>) were infected with RSV (1 ml in maintenance medium) (Edinburgh strain) [Ogilvie *et al.*, 1981] at multiplicity of infection of 2-3 infectious particles per cell for 60 min. The medium was replaced and incubated with 10 ml maintenance medium overnight at 37°C.

Cultures were rinsed with PBS and 1 ml per flask of ethylenediaminetetraacetic acid (EDTA) (0.05%) was added at 37°C for 10 min. Maintenance medium (5) ml was added to counteract the effect of EDTA. The suspension was centrifuged at 700 g for 10 min and the cells were resuspended in maintenance medium. The cell count was adjusted to 10<sup>6</sup> cells/ml using a Neubauer haemocytometer.

#### **7.2.4 Bacterial binding to HEp-2 cells in suspension**

The bacterial suspension was labelled with FITC and the binding assays were performed as described in chapter 3. For *S. aureus*, the attachment of bacteria to RSV-infected HEp-2 cells and uninfected cells was determined using 160 bacteria : cell, 320 bacteria : cell and 640 bacteria : cell at two incubation periods, 30 and 120 min. For *B. pertussis* ratios of bacteria:cell ranging from 400-6400 were tested at an incubation time of 120 min. The proportion of RSV-infected cells was determined by flow cytometry [Raza *et al.*, 1993]. Mouse monoclonal antibody to RSV-F protein was reacted with RSV-infected cells. The cells were washed and 20 µl of FITC-labelled anti-mouse IgG antibody (Sigma) was added and incubated for 30 min at 37°C. The rest of experiment was carried out as described in 4.2.3.



### **7.2.5 Analysis of binding by flow cytometry**

Each sample was analysed by flow cytometry as described above (3.2.3.4). Immunoanalysis was used in the analysis and the binding index (BI) of each sample was determined.

### **7.2.6 Statistical methods**

The analyses were carried out on the logarithms of the binding indices and a three-factor analysis of variance was used to test whether RSV infection was related to bacterial binding to HEp-2 cells at two different concentrations of bacteria per cell.

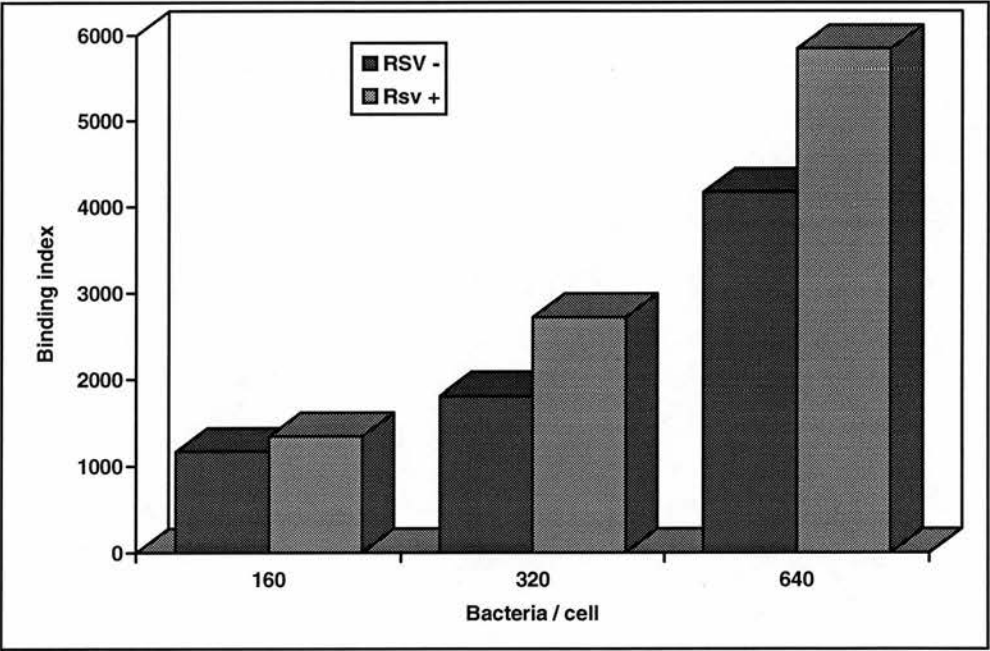
## **7.3 Results**

### **7.3.1 Binding of bacteria to RSV infected HEp-2 cell**

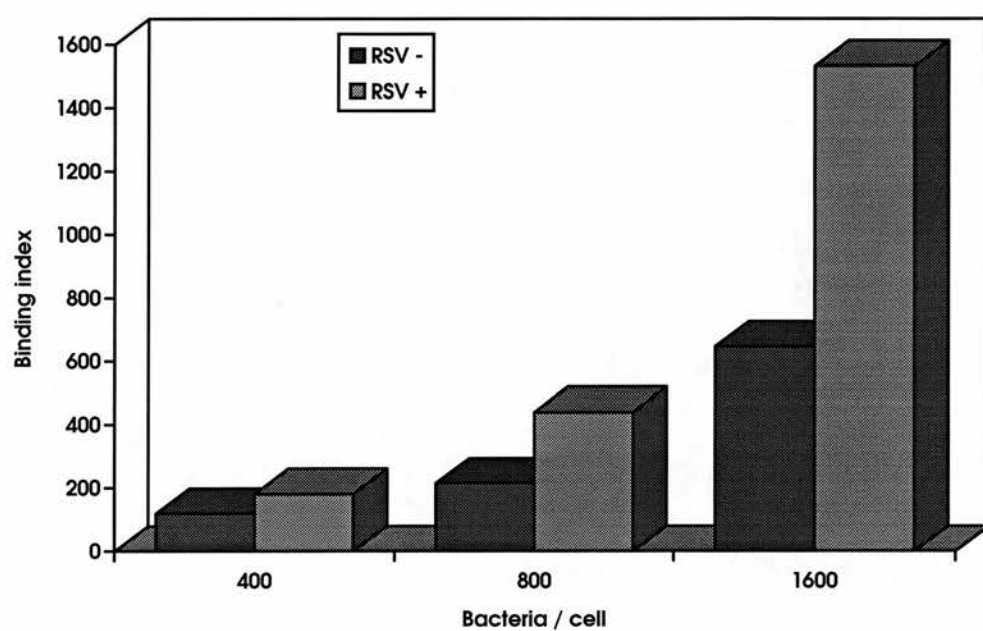
The percentage of RSV-infected cells was determined at the time of the attachment assay by flow cytometry. The proportion of cells binding the specific anti-F antibody was > 80% for each experiment.

#### **7.3.1.1 Dose response**

The effect of RSV infection on the attachment of *S. aureus* strain NCTC 10655 to HEp-2 cells is shown in (Figure 7.1). The infected cells bound more staphylococci than the uninfected cells at both concentrations of bacteria used (320 and 640 bacteria/cell) and it was reflected in both the percentage and the mean. *B. pertussis* strain 8002 gave similar pattern (Figure 7.2).



**Figure 7.1 Binding of *S. aureus* strain NCTC 10655 to HEp-2 cells and HEp-2 cells infected with RSV.**



**Figure 7.2 Binding of *B. pertussis* strain 8002 to HEp-2 cells and HEp-2 cells infected with RSV.**

### 7.3.1.2 Time course

The attachment of *S. aureus* strain NCTC 10655 to HEp-2 cells was measured using 320 and 640 bacteria / cell at two incubation periods (30 min and 120 min) (Figure 7.3). Binding increases over 120 min, but the incubation at 30 min gave a reasonable attachment and this incubation time was used for the further experiments. The incubation time for attachment of *B. pertussis* was carried out at 120 min. as it was the optimal time for the binding with the epithelial cells in a previous study (6.3.2).

### 7.3.1.3 Effect of RSV infection

Statistics were performed on the results of seven experiments on binding of *S. aureus* strain NCTC 10655 to RSV infected HEp-2 cells compared with binding to uninfected cells. The binding indices for the RSV-infected cells were greater than for the uninfected cells :  $P < 0.05$ , 95% CI 109-155%, after adjusting for the effect of bacterial concentration on binding (Figure 7.4).

The binding indices for *B. pertussis* strain 8002 to RSV infected cells were greater than for the uninfected cells for all the ratios of bacteria per cell used (Figure 7.5), and strain 250815 gave a similar pattern (Figure 7.6). For each strain there was a similar pattern for two tests performed on two different days.

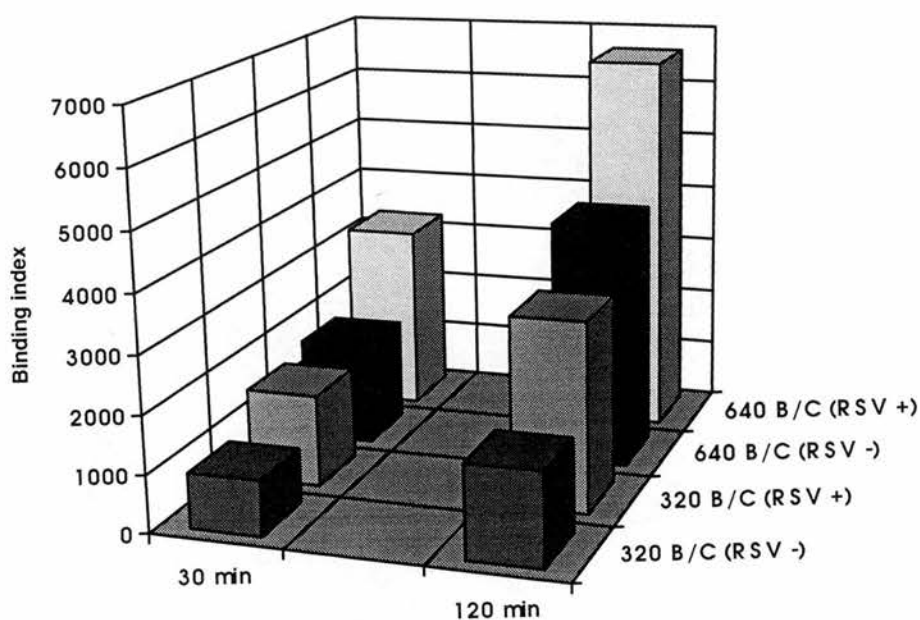


Figure 7.3 Binding of *S. aureus* strain 10655 to HEP-2 cells and HEP-2 cells infected with RSV at 30 and 120 min (B/C = bacteria/cell).

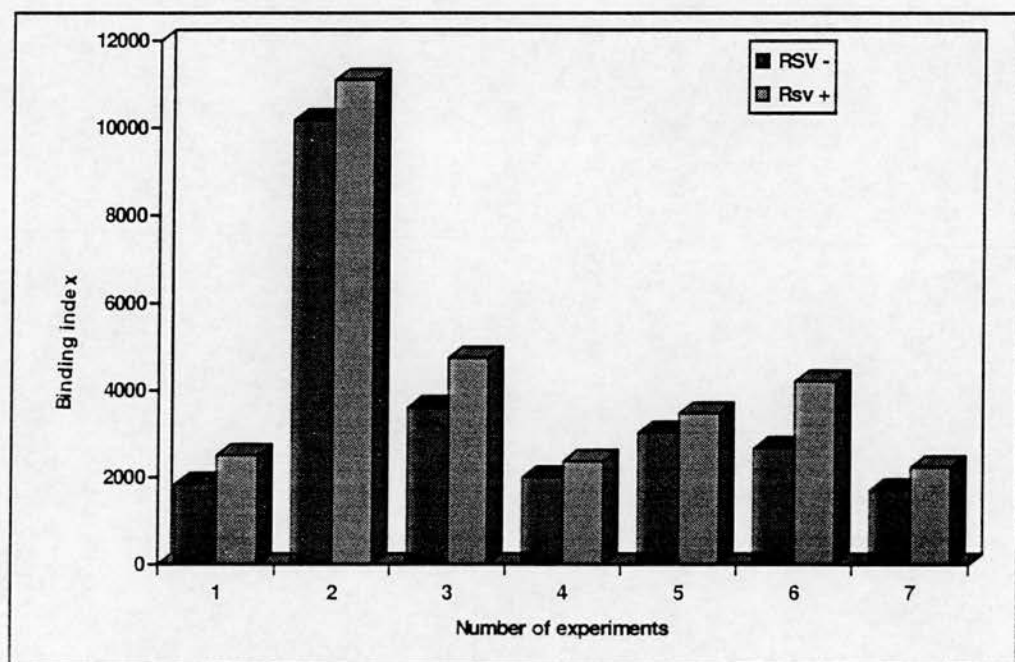


Figure 7.4 Binding of *S. aureus* strain NCTC 10655 to HEp-2 cells and HEp-2 cells infected with RSV (640 bacteria : cell).

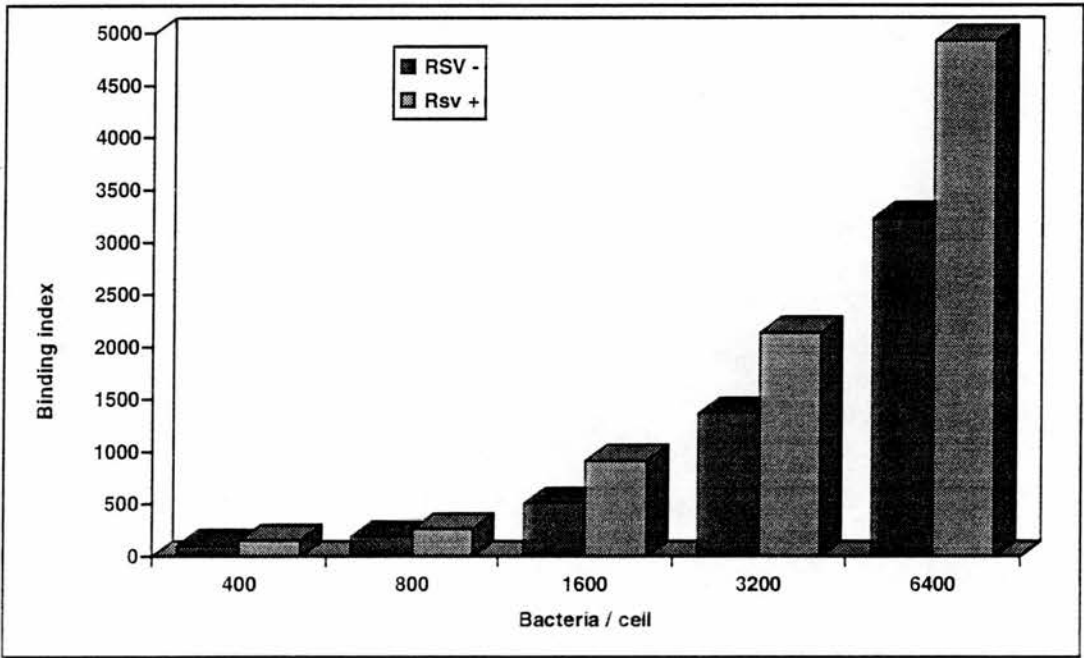


Figure 7.5 Dose response of binding of *B. pertussis* strain (8002) to HEP-2 cells or RSV infected HEP-cells.

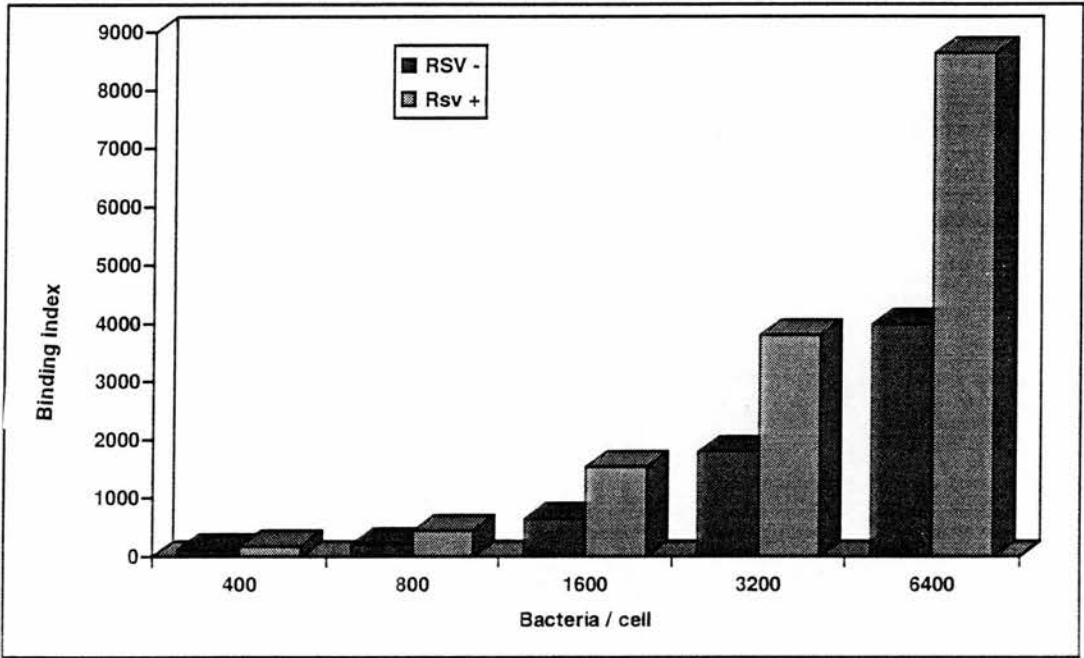


Figure 7.6 Binding of *B. pertussis* strain (250815) to HEp-2 cells or RSV infected HEp-2 cells with different ratios of bacteria / cell.



## 7.4 Discussion

For many years it has been recognised that viral infection might increase susceptibility to secondary bacterial infection [Nichol and Cherry 1967]. Viral infections have been shown to enhance carriage of staphylococci [Ramirez-Ronda *et al.*, 1981] and toxic shock syndrome caused by toxin producing staphylococci has been identified as a complication of influenza or influenza like-illness. [McDonald *et al.*, 1987]. Binding of both meningococci and type b *Haemophilus influenzae* to tissue culture cells derived from human epithelium (HEp-2) was significantly increased when the cells were infected with RSV [Raza *et al.*, 1993]; and, in this study, both the toxigenic strain of *S. aureus* and the two strains of *B. pertussis* tested bound in greater numbers to the RSV-infected cells. If there are similar interactions *in vivo* infection with RSV, common during the first year of life, might also enhance colonization by these bacteria.

Two hypothesis have been proposed to explain why virus infected cells bind more bacteria: virus infection upregulates expression of host cell surface antigens that can act as receptors for bacteria; new antigens coded for by RSV expressed on the surface of infected host cells can act as additional receptors for bacteria [*e.g.*, the F (fusion) and G (adhesion) glycoproteins]. There is evidence to suggest both hypotheses.

Viral infections induce  $\gamma$ -interferon which enhances expression of CD14 to which bacterial endotoxins bind. RSV-infected HEp-2 cells bind more monoclonal antibody to CD14 than the uninfected cells (Raza *et al.*, 1994). The expression of CD14 and binding of endotoxin to cultured monocytes was enhanced by pretreatment of the cells with interferon [Jackson *et al.*, 1993]. This might be one of the explanations for the enhanced binding of the *B. pertussis* to the RSV infected cells as these bacteria produce endotoxin. RSV infection

also enhanced expression of Le<sup>x</sup> on HEp-2 cells and anti-Le<sup>x</sup> significantly inhibited the binding of *S. aureus* and *B. pertussis* to epithelial cells.

There is evidence that glycoprotein G (but not glycoprotein F) of RSV acts as an additional receptor for meningococci. Future work with this model will explain the roles of CD14, Le<sup>x</sup> and glycoprotein G as receptors for *B. pertussis* and *S. aureus*.

# Chapter 8

## General Discussion

The aims of this chapter are to examine the results obtained in the previous chapters in the context of the original objectives of the project; to highlight the applications or limitations of the experiments and methods of analysis used throughout this study; and to suggest future work.

### 8.1 Objectives of the project

The results of this thesis provided answers for the questions to be investigated. The first objective was to test the hypothesis that Le<sup>a</sup> antigen is a receptor on epithelial cells for toxigenic bacteria such as *S. aureus* and *B. pertussis*. As secretors were suggested to have lower levels of Le<sup>a</sup> on their cells compared with non-secretors, binding of staphylococci to cells from secretor and non-secretor donors was examined. There was not a clear difference in binding of all strains of staphylococci to cells of secretors and non-secretors. Assessment of binding of anti-Le<sup>a</sup> to BEC of donors helped resolve these observations. The amount of Le<sup>a</sup> on the cells of secretors was highly variable, some bind as much anti-Le<sup>a</sup> as cells of non-secretors (Figure 3.3). Inhibition of binding by pretreatment of BEC with anti-Le<sup>a</sup> and correlation between bacterial binding and amount of anti-Le<sup>a</sup> bound to cells provided additional evidence that Le<sup>a</sup> could be a receptor for *S. aureus*. Subsequent isolation of the bacterial cell wall adhesin by Synsorb-Le<sup>a</sup> and complementary studies in which anti-idiotypic antibodies to monoclonal anti-Le<sup>a</sup> were found to agglutinate all

staphylococcal strains tested were further evidence for the hypothesis [Essery *et al.*, 1994b].

The second objective was to test the effect of RSV infection on binding to epithelial cells. Binding of both *S. aureus* and *B. pertussis* was greater for RSV infected cells. While virus infection might not be a direct cause of SIDS, it might contribute to colonization by potentially pathogenic bacteria.

## **8.2 Applications and limitations of methods and results**

### **8.2.1 Assessment of bacterial binding by flow cytometry**

One of the advantages of flow cytometry is the ability to analyse large numbers of cells in a short time. Direct labelling of bacteria with FITC was used for the study. Some workers have used fluorescent antibodies to identify attached or suspended bacteria [Donnelly and Baiegant, 1986]; however such an approach might alter the viability or receptor properties of bacteria before exposure to eukaryotic cells. Direct labelling of *S. aureus* with FITC did not affect their binding to BEC as assessment of binding by ELISA showed a correlation between the number of bacteria added to the wells and the OD reading for both FITC-labelled and unlabelled *S. aureus*. Direct visualisation of the bacteria by confocal microscopy confirmed that the labelled bacteria were bound to the cells.

Binding indices were used to quantify the bacterial binding to cells. When the ratio of bacteria per cell was increased from a lower to a higher level, the values for both the percentage of positive cells and the mean fluorescence were increased. In contrast, when the majority of cells bound bacteria the difference in bacterial binding between 2

populations was clearer from values obtained for the mean. For the above reasons binding indices were used to compare bacterial binding among individuals.

### **8.2.2 Inhibition of binding of *S. aureus* to BEC**

Rosenstein *et al.* [1988] have reported that fucosylating the precursor type 1 to H and Le<sup>b</sup> structures interfered with the binding of *E. coli*. There was a correlation of binding between binding of a toxigenic strain of *S. aureus* to BEC from secretors and non-secretors and binding of anti-Le<sup>a</sup> to their cells. Although anti-precursor type 1 and anti-Le<sup>x</sup> significantly inhibited binding of bacteria, there was no correlation between binding of bacteria and binding of these monoclonals to BEC. As binding of monoclonal anti-precursor type 1 was not detected on BEC of all individuals regardless of their secretor status, this suggests that precursor type 1 could be an additional site of recognition for the bacterial adhesins rather than the major receptor. The possibility that the anti-precursor type 1 and anti-Le<sup>x</sup> antibodies are binding to epitopes on structurally similar molecules must also be considered.

Inhibition of bacterial binding by treatment of the target cells is always subject to criticism that the antibodies are binding an epitope near the receptor and blocking access of the bacteria. Experiments with either monoclonal and polyclonal antibodies are limited by this potential problem. The subsequent use of anti-idiotypic antibodies to monoclonal Le<sup>a</sup> supports the results of the inhibition studies with anti-Le<sup>a</sup>. Attempts to produce anti-idiotypic antibodies to monoclonal anti-Le<sup>x</sup> or anti-precursor type 1 have been limited by the cost of the monoclonal antibodies.

### **8.2.3 Detection of Lewis antigens by flow cytometry**

The results obtained by ELISA for Le<sup>a</sup> in secretions agreed with the detection of the antigen on BEC by flow cytometry. BEC of secretors bound anti-Le<sup>a</sup> and there was greater variability among results for the secretors compared with non-secretors.

There was much lower binding of the antibody to cells from Lewis negative individuals whose cells were not agglutinated with anti-Le<sup>a</sup> or anti-Le<sup>b</sup>. These results suggest the greater sensitivity of flow cytometry can detect small amounts of Le<sup>a</sup> or cross reactive antigens on their cells which are insufficient for detection by the haemagglutination reaction.

The flow cytometry method depends on the specificity of antibodies used to detect the antigens of interest. Monoclonal antibodies are better than polyclonal ones for these assays; however, if several monoclonals to a particular antigen are available, it would be advisable to compare each for specificity with known positive and negative controls before screening large numbers of samples.

### **8.2.4 Application of Synsorb-Le<sup>a</sup> to isolation of the adhesin from *S. aureus***

Direct evidence for a specific receptor such as Le<sup>a</sup> makes it easier to isolate and purify the bacterial adhesins. The Synsorb Le<sup>a</sup> bound a protein from cell wall preparations of a toxigenic strain of *S. aureus*, the binding of which was inhibited by treatment of BEC with anti-Le<sup>a</sup>; and this purified protein was able to block adherence of the bacteria to BEC. This strain was also agglutinated by anti-idiotypic antibodies produced against monoclonal



anti-Le<sup>a</sup> [Essery *et al.*, 1994b]. The method might be applied to isolation of adhesins from other bacteria or yeasts to which non-secretors appear to be at increased risk of infection.

### **8.3 The role of RSV in SIDS**

RSV is a major contributor to winter peaks of infant deaths due to lower respiratory tract infection [Anderson *et al.*, 1989]. These infections are associated with apnoea in young infants [Anas *et al.*, 1982]. Although there is no evidence that SIDS is caused directly by RSV infection, the virus might enhance colonization by potentially pathogenic bacteria. In addition to the native receptors for bacteria on the host cells, there is evidence that new receptors for bacteria may be induced on virus-infected epithelial cells. The mechanisms by which viruses enhance bacterial binding is unclear and is beyond the objectives of this thesis; however, work by colleagues in the laboratory have identified 2 mechanisms by which RSV infection enhances bacterial binding: 1) The glycoprotein G encoded by RSV might be an additional receptor for bacteria; 2) RSV infection upregulates expression of host cell antigens to which bacteria bind CD15 (Le<sup>x</sup>), CD14 and CD18 [Raza *et al.*, 1994]. Further studies will assess the effect of antibodies to the G glycoprotein of RSV, Le<sup>x</sup>, CD14 and CD18 on binding of staphylococci and pertussis to virus infected cells. Virus infection might also potentiate the effect of bacterial toxins, both endotoxins and exotoxins, as suggested by the work of Jakeman *et al.* [1991]. Interferon  $\gamma$  induced by virus infection enhances the endotoxin receptor (CD14) on monocytes [Jackson *et al.*, 1993]. If release of inflammatory mediators in response to endotoxin is dose dependent, this might enhance the release of these products by enhancing binding of endotoxin (Figure 8.1).

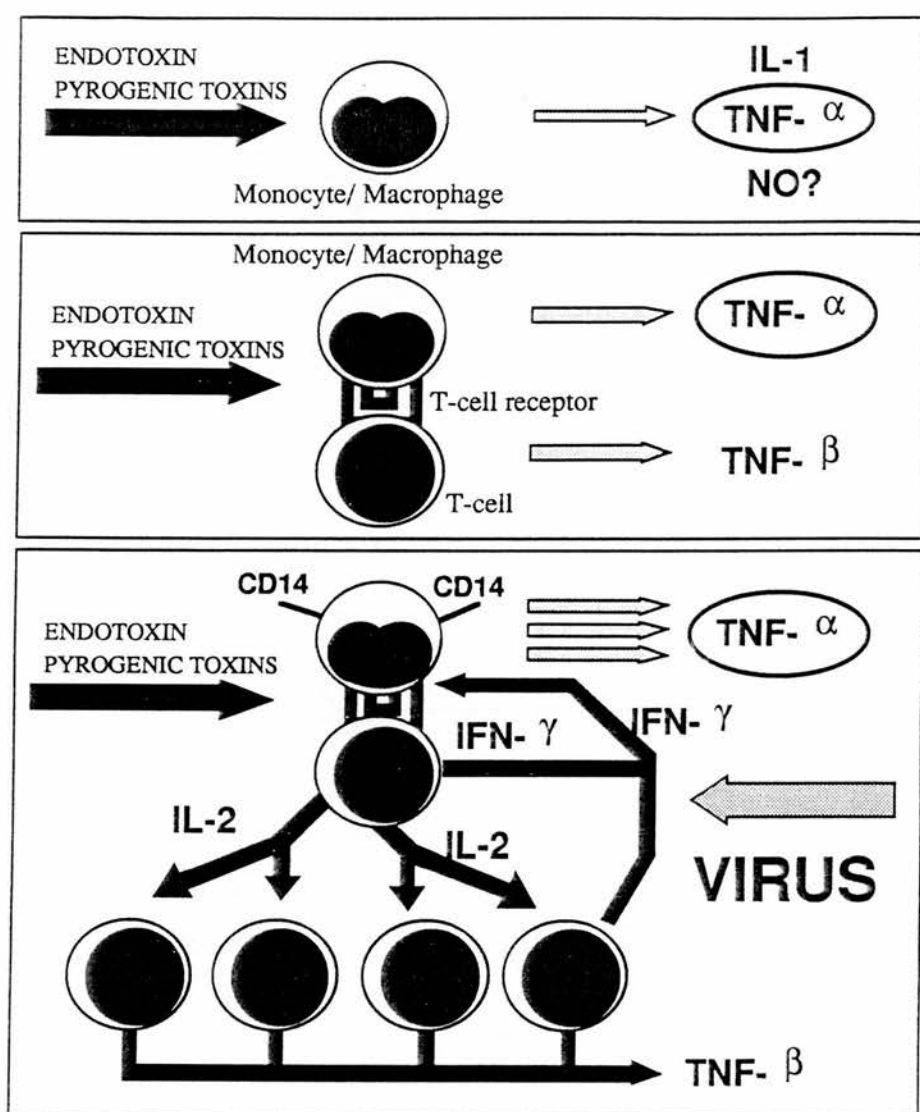


Figure 8.1 Synergy between viral infection, endotoxins and pyrogenic toxins resulting in release of inflammatory mediators.



## 8.4 The role of DPT immunization in SIDS

Results from this study indicate that *B. pertussis* and *S. aureus* have adhesins that bind to Le<sup>a</sup> and Le<sup>x</sup> on epithelial cells. The pertussis toxin and the staphylococcal enterotoxin B both bind to Le<sup>a</sup> and Le<sup>x</sup>. If the epitopes on the adhesins and/or toxins that bind to Le<sup>a</sup> and Le<sup>x</sup> are structurally similar, immunization of infants against pertussis might induce antibodies which could neutralize the staphylococcal antigens. Evidence from this laboratory indicates that there is significant binding of anti-pertussis toxin to some of the staphylococcal pyrogenic toxins [Essery *et al.*, 1994b].

The age distribution of SIDS is associated with the time of immunization with DPT and there are reports of SIDS occurring 24 hours after receiving the first dose of DPT [Bernier *et al.*, 1982; Baraff *et al.*, 1983]. A number of epidemiological studies have indicated that SIDS cannot be attributed to DPT immunization [Emery, 1981; Hoffman *et al.*, 1987]. A study by Walker *et al.* [1987] indicated that there was a six fold increased risk of SIDS in unimmunised infants compared to immunised infants. If DPT immunization induces antibodies that neutralize other toxins, earlier immunization might contribute to the recent decrease in SIDS in Scotland since 1990.

## 8.5 Bacterial toxins and SIDS

Figure 8.2 summarizes the sequence of events that might lead to SIDS. The work in this thesis has identified Le<sup>a</sup> antigen and RSV infection as two factors that might enhance colonization of infants by *B. pertussis* and *S. aureus*. Mothers' smoking is another factor

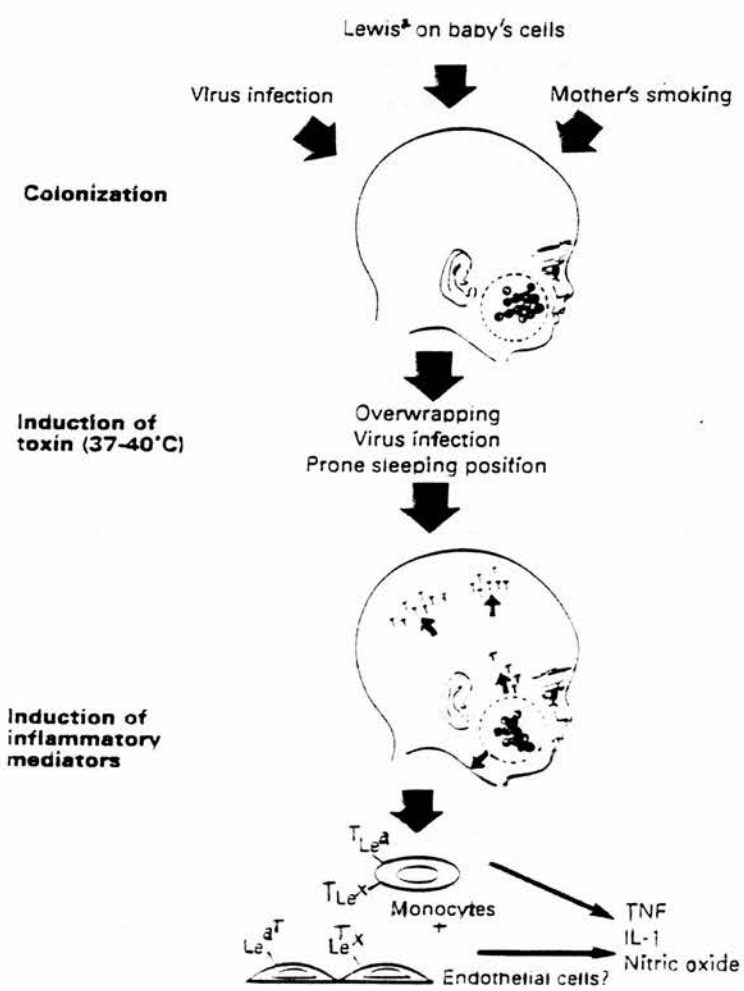


Figure 8.2 Possible role of pyrogenic toxins in SIDS.

that might enhance bacterial colonization and it is significantly associated with SIDS. Recent studies using the methods developed for bacterial binding studies have found significantly increased binding of *S. aureus* to cells of smokers compared to cells of non-smokers [Saadi *et al.*, 1994].

Not all strains of staphylococci which colonize the epithelial cells are toxigenic, and not every infant who is colonized by toxigenic staphylococci will become a SIDS victim. There have to be additional factors that precipitate the chain of events leading to cot death. These toxins are only produced between 37 - 40°C and the temperature of the mucosal surfaces are usually below the permissive temperature due to passage of air during breathing. The infant's body temperature might be increased by concurrent minor respiratory infection and this effect could be increased by over-wrapping [Fleming *et al.*, 1990] or placing the infant in the prone position [Gilbert *et al.*, 1992] which might reduce heat loss through the face or block a nostril creating a "micro-environment" in which the permissive temperature is reached. Pyrogenic toxins are thought to act directly on the brain to raise the temperature. This could result in amplification of toxin production as greater amounts are present at 40°C than at 37°C.

The mechanisms by which the microorganisms might cause sudden death are summarised in Figures 8.3. The pyrogenic toxins are superantigens that induce inflammatory mediators via their action on monocytes and T cells (*e.g.*, TNF, nitric oxide and IL-1 (Figure 8.3). The effect of respiratory infection, increasing the duration of sleep apnoea, and increasing the risk of SIDS, could be mediated through the somnogenic effects of IL-1 [Guntheroth, 1989]. There is no direct biochemical evidence that IL-1 is increased in SIDS's sera; however, systemic release of IL-1 causes disturbances of hormonal control [Cunningham and de Souza, 1993]; and Naeye *et al.* [1980] have reported an increase in the level of

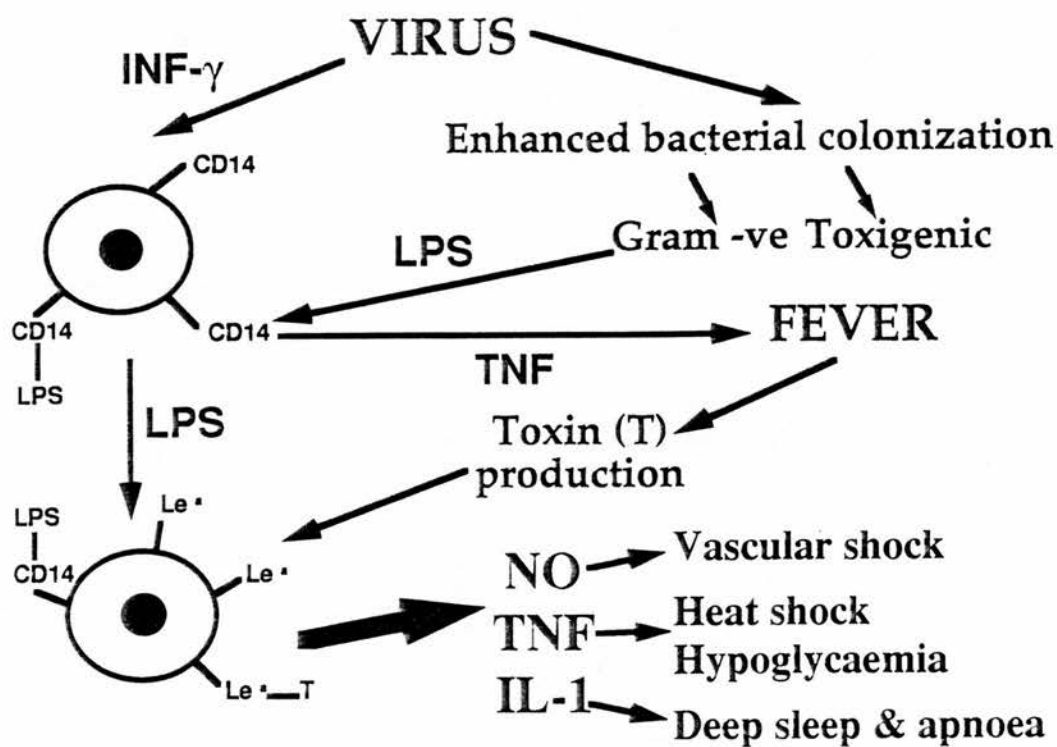


Figure 8.3 Summary of mechanisms by which microorganisms might cause sudden death.

serum cortisol among SIDS victims. The actions of IL-1 include: induction of fever; increased the production of TNF, IL-6 and IL-8 [Kluger, 1991]; and induction of haemodynamic shock [Ikejima *et al.*, 1989].

TNF might induce heat shock and is probably a major factor in the induction of toxic shock. It can also cause severe hypoglycaemia which could result in glucose starvation in the brain [Stevens *et al.*, 1993]. The pyrogenic toxins can enhance the effect of endotoxin by 100,000 fold [Bohach *et al.*, 1990]. The scheme outlined in Figure 8.3 could explain the synergistic effects of *S. aureus* exotoxins and endotoxins of *E. coli* in the chick embryo model [Drucker *et al.*, 1992].

## **8.6 Prophylaxis of SIDS**

The preventive measures currently recommended fit the hypothesis proposed for SIDS. Discouraging the prone sleeping position and measures to avoid over heating babies might reduce the probability that the toxins are induced. Discouraging smoking among mothers and other people living with the infant will reduce risk of respiratory infection. Encouragement of breast feeding will reduce risk of infection. Infants who experienced apnoea should be monitored and examined for carriage of toxigenic bacteria. Early immunization for DPT might induce antibodies that reduce colonization or partially neutralize toxins.

## **8.7 Further studies**

It is important to test the immunogenicity of the adhesins that bind Le<sup>a</sup>. ELISA techniques will be used to determine if there is an immune reaction to these adhesins in the sera and

secretions of SIDS victims compared with healthy infants and adults. Assessment of human milk for antibodies or glycoconjugates that inhibit the adhesin could help explain the protective effect of breast feeding. Polyclonal and monoclonal antibodies to the adhesins can be produced and used to screen the bacteria isolated from the SIDS victims and healthy infants for the expression of the adhesins. The antibodies can be tested in attachment assays to determine if they reduce bacterial binding.

Epidemiological studies have been initiated to determine if factors associated with enhanced binding of *B. pertussis* and *S. aureus* (expression of Le<sup>a</sup>, RSV infection, mother smoking) are reflected in isolation of potentially pathogenic bacteria from healthy infants. These studies combined with laboratory investigations on the increased binding of staphylococci and pertussis to RSV-infected cells will provide insights into the associations between minor respiratory infection and SIDS.

The hypothesis proposed can be tested, but it will require cooperative studies between research workers in a variety of related disciplines, microbiology, immunology, developmental physiology and epidemiology.

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## Factors enhancing adherence of toxigenic *Staphylococcus aureus* to epithelial cells and their possible role in sudden infant death syndrome

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### SUMMARY

Toxigenic strains of *Staphylococcus aureus* have been suggested to play a role in sudden infant death syndrome (SIDS). In this study we examined two factors that might enhance binding of toxigenic staphylococci to epithelial cells of infants in the age range in which cot deaths are prevalent: expression of the Lewis<sup>a</sup> antigen and infection with respiratory syncytial virus (RSV). By flow cytometry we demonstrated that binding of three toxigenic strains of *S. aureus* to cells from non-secretors was significantly greater than to cells of secretors. Pre-treatment of epithelial cells with monoclonal anti-Lewis<sup>a</sup> or anti-type-1 precursor significantly reduced bacterial binding ( $P < 0.01$ ); however, attachment of the bacteria correlated only with the amount of Lewis<sup>a</sup> antigen detected on the cells ( $P < 0.01$ ). HEp-2 cells infected with RSV bound significantly more bacteria than uninfected cells. These findings are discussed in context of factors previously associated with SIDS (mother's smoking, bottle feeding and the prone sleeping position), and a hypothesis proposed to explain some cases of SIDS.

### INTRODUCTION

The suggestion that microorganisms are involved in the aetiology of some cases of sudden infant death syndrome (SIDS) is based on evidence from a variety of epidemiological and pathological studies. SIDS occurs during the period when maternal antibodies have declined and the infant immune system is immature. There is a marked seasonal variation in SIDS; the risk increases in autumn and winter when respiratory infections are more common [1, 2]. There is often a history of upper respiratory tract infection in these infants [3]. Mother's smoking was identified as a factor for SIDS in the New Zealand studies [4]. Smoking and passive exposure to cigarette smoke have been associated with increased risk of respiratory tract infections [5] and with carriage of potentially pathogenic microorganisms [6, 7]. At post-mortem examination, there is often evidence of

minor inflammation and infection of the respiratory tract in many SIDS infants [8].

There are conflicting results on the role of viruses [9, 10] and toxigenic intestinal bacteria in SIDS [11-15]. Recently, it has been suggested that nasopharyngeal colonization by toxigenic strains of *Staphylococcus aureus*, particularly those producing the toxic shock syndrome toxin 1 (TSST-1), might be involved in some of these infant deaths [16]. Pyrogenic toxins are produced by many strains of *S. aureus*, and similar ones are found among some strains of group A  $\beta$ -haemolytic *Streptococcus pyogenes*. These are 'superantigens' that have significant physiological effects such as induction of fever ( $> 38^{\circ}\text{C}$ ), possibly by direct action on the hypothalamus or by their induction of tumor necrosis factor (TNF) and interleukin-1 by monocytes [17]. *S. aureus* was isolated from a higher proportion of SIDS infants (41.3%), compared with a control population (28.3%) [18]. In this study we examined two factors that might enhance density of colonization by toxigenic staphylococci in infants during the period when they are at risk of SIDS: expression of the Lewis<sup>a</sup> blood group antigen, and infection with respiratory syncytial virus (RSV).

Non-secretion, the genetically controlled inability of an individual to secrete the glycoprotein form of his/her ABO blood group antigens, is associated with susceptibility to a number of bacterial diseases and with asymptomatic carriage of some potentially pathogenic microorganisms [19], including group A streptococci [20]. The secretor gene also influences expression of the Lewis blood group antigens; non-secretors produce only Lewis<sup>a</sup>; secretors produce Lewis<sup>b</sup> predominantly but also variable amounts of Lewis<sup>a</sup>. The amount of Lewis<sup>a</sup> present in secretors depends on the relative activities of the fucosyl transferases coded for by the secretor gene and the Lewis gene. If the secretor fucosyl transferase acts first on the precursor chain, the Lewis enzyme can add fucose to the subterminal sugar of the chain to produce Lewis<sup>b</sup>. If the Lewis fucosyl transferase acts on the precursor first, the Lewis<sup>a</sup> molecule cannot act as a substrate for the secretor enzyme.

One of the hypotheses proposed to explain the increased proportion of non-secretors among carriers of some potentially pathogenic bacteria and yeasts is that the Lewis<sup>a</sup> antigen is one of the host cell receptors for some microorganisms [19, 21, 22]. In this context, the reported high proportion of infants expressing Lewis antigens during the first year of life was of particular interest [23]. Among infants, the enzyme coded for by the secretor gene appears to be less efficient than that coded for by the Lewis gene. Although the majority of infants are secretors (75-80%), during the first year of life they will express easily detectable amounts of Lewis<sup>a</sup>. The peak in the proportion of infants expressing Lewis<sup>a</sup> is 2-4 months, similar to the highest incidence of cot deaths.

It has been reported that both natural and experimental viral infections enhance colonization by *S. aureus* [24]. RSV infected cells bind greater numbers of *Neisseria meningitidis* and type b *Haemophilus influenzae* compared with uninfected cells [25]. As RSV infects almost half of all infants by the age of 12 months and is most prevalent in the winter months [26], it is an obvious candidate for investigation of the role viruses might play in enhanced colonization by toxigenic bacteria.



These observations prompted the following questions:

1. Is Lewis<sup>a</sup> detected in SIDS infants?
2. Do strains of toxigenic *S. aureus* bind in greater numbers to non-secretor cells expressing larger amounts of Lewis<sup>a</sup>?
3. As some viral infections increase carriage of staphylococci, do tissue culture cells infected with RSV bind greater numbers of staphylococci than uninfected cells?

The results of these investigations are discussed in the context of epidemiological data on the role of microorganisms in SIDS.

#### SUBJECTS AND METHODS

Respiratory tract secretions (89) obtained from SIDS infants during autopsy were provided by Dr J. N. Inglis and Dr P. Molyneaux (Regional Virus Laboratory, City Hospital, Edinburgh) and by Dr A. Gibson (Royal Hospital for Sick Children, Glasgow). These were examined for presence of H [27] and Lewis antigens [28] by enzyme-linked immunoassays (ELISA) described previously.

*S. aureus* strains NCTC 10652, NCTC 10654, NCTC 10655, NCTC 10656, NCTC 10657, NCTC 11965, and NCTC 8532 were obtained from Dr A. Wieneke Central Public Health Laboratory, Colindale. Strains 40654 and 41206 were kindly provided by Dr J. Medcraft, Public Health Laboratory Service, Department of Microbiology, Reading, Berkshire. The strains and the toxins they produced are listed in Table 1. The bacteria were grown on nutrient agar or for some experiments on blood agar to examine the effect of medium on binding to epithelial cells.

Buccal epithelial cells (BEC) were obtained from pairs of healthy secretor and non-secretor donors matched as closely as possible for ABO blood group, age and sex. ABO groups of the donors were determined by slide agglutination of erythrocytes with monoclonal anti-A and anti-B antibodies (Scottish National Blood Transfusion Service). Secretor status was determined from saliva by haemagglutination inhibition assays [29], initially confirmed by tube agglutination with monoclonal anti-Lewis<sup>a</sup> or anti-Lewis<sup>b</sup> antibodies (Scottish National Blood Transfusion Service) and later by ELISAs for H and Lewis antigens.

BEC were collected by rubbing the inside of the cheeks with cotton swabs. To remove the cells, the swabs were agitated in 10 ml phosphate-buffered saline (PBS) (pH 7.2). They were washed twice in PBS in a Sorvall RT 6000 centrifuge (300 g for 10 min) and the concentration adjusted to  $2 \times 10^5$  ml<sup>-1</sup> after determination of the number of cells microscopically in an improved Neubauer haemocytometer.

Bacteria were labelled with fluorescein isothiocyanate (FITC) by a modification of the method of Wright and Jong [30]. A heavy suspension of bacteria in PBS was prepared and washed twice by centrifugation at 1000 g for 20 min. The bacterial pellet was resuspended in 4 ml of a freshly prepared 0.04% solution of FITC in sodium carbonate (0.05 M) and sodium chloride (0.1 M) (pH 9.2). The mixture was incubated at 37 °C for 20 min and washed twice with PBS. The pellet was

resuspended in PBS and filtered through a Millipore membrane filter (5  $\mu$ m pore size) to remove clumps of bacteria.

The bacterial concentration was determined by measuring the optical density of the suspension at 540 nm. The linear relationship between optical density and total count was determined for each strain.

#### *Binding of bacteria to BEC of secretors and non-secretors*

BEC (200  $\mu$ l) were mixed with 200  $\mu$ l of the FITC-labelled bacteria at the following ratios of bacteria per cell: 80:1, 160:1 and 320:1. The mixtures were incubated at 37 °C for 30 min with gentle shaking in an orbital incubator (Gallenkamp). The cells were washed twice with PBS by centrifugation at 300 g for 10 min to remove unattached bacteria. The samples were resuspended in 300  $\mu$ l 1% buffered paraformaldehyde (Sigma) and stored in the dark at 4 °C until analysed.

#### *Analysis of binding assays*

Each sample was analysed by flow cytometry with an EPICS-C (Coulter Electronics, Luton, UK) equipped with a 5 W laser with a power output of 200 MW at 488 nm. Cells were selected from a display of forward angle light scatter (size) versus 90° light scatter (granularity) by means of a bitmap. The bitmap included the main population of the cells and excluded debris and clumps from further analysis. The percentage of cells with fluorescence greater than background level was recorded on a one-parameter histogram which measured fluorescence on a logarithmic scale. The mean fluorescence channel values for positive cells were obtained from a one-parameter histogram measuring fluorescence on a linear scale. The results were analysed by Immunoanalysis (Coulter), a computer programme that subtracts the values of the control population from the test population at each channel of the two histograms. The binding index (BI) of each sample was calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel value.

#### *Inhibition of bacterial binding*

BEC obtained from secretors or non-secretors were treated with monoclonal anti-Lewis<sup>a</sup> (LM112/161.8, kindly supplied by Dr R. H. Fraser, Law Hospital, Carlisle) or monoclonal antibody to the type 1 precursor chain (anti-precursor type 1) (clone no 619/102, Russel Fine Chemicals, Chester) for 60 min at 37 °C. After washing twice by centrifugation at 300 g for 10 min, the attachment assay was performed and analysed as described above.

#### *Binding of bacteria to HEp-2 cells*

Methods for assessment of binding of *S. aureus* strains NCTC 8532 and NCTC 10655 to uninfected and RSV-infected HEp-2 tissue culture cells and analysis of results were as described for *H. influenzae* [25].

#### *Detection of Lewis<sup>a</sup> and type 1 precursor antigens on BEC of secretor and non-secretor donors*

Buccal epithelial cells (200  $\mu$ l,  $2 \times 10^5$  ml<sup>-1</sup>) from 7 secretors, 7 non-secretors and 3 secretor donors whose erythrocytes were not agglutinated by either anti-Lewis<sup>a</sup>

or anti-Lewis<sup>b</sup> monoclonal antibodies (Scottish National Blood Transfusion Service) (Lewis-negative individuals) were incubated with 200  $\mu$ l of monoclonal antibodies to Lewis<sup>a</sup> (1/5) or precursor type 1 (1/10) for 60 min at 37 °C. The cells were washed twice with PBS at 300 g for 10 min and incubated with 200  $\mu$ l rabbit anti-mouse IgM conjugated with FITC (1/200) (Sigma). The FITC-labelled antibody was also added to 200  $\mu$ l of cells which had not been treated with the first antibody as a control. After 30 min incubation in an orbital shaker at 37 °C, the cells were washed twice with PBS. They were resuspended in 300  $\mu$ l 1% buffered paraformaldehyde and stored in the dark at 4 °C until analysed. The cells were analysed on an EPICS-C flow cytometer as described above and the binding indices calculated as before.

#### *Statistical methods*

All analyses were carried out on the logarithms of the binding indices which conformed more closely to a normal distribution than the raw values. Differences between groups were tested by paired or unpaired *t* tests as appropriate, and confidence limits for the mean values in one group were expressed as a percentage of those in the other by taking antilogarithms. Analysis of covariance was used to test whether binding levels were associated with the amount of anti-Lewis<sup>a</sup> in secretors and non-secretors tested on different days, while the association in a similar experiment with anti-precursor on a single day was tested by Pearson correlation. Three-factor analysis of variance was used to test whether RSV infection was related to binding of HEP2 cells at two different concentrations of bacteria per cell.

### RESULTS

#### *Detection of Lewis<sup>a</sup> antigen in body fluids of SIDS infants*

Lewis<sup>a</sup> antigen was detected in 63/89 (71%) of the specimens from SIDS infants.

#### *Binding of S. aureus to epithelial cells of secretors and non-secretors*

The results of the binding assays are presented in Table 1 as the 95% confidence limits for binding of the bacteria to non-secretor cells expressed as a percentage of binding to cells from matched secretors. Confidence intervals for which both values are above 100 indicated significant evidence that cells from non-secretors bound more staphylococci than cells from secretors.

There was no significant difference in binding to cells of non-secretors compared with binding to cells of secretors observed for the non-toxigenic strain NCTC 8532 or five of the toxin producing strains: NCTC 41206, NCTC 40654, NCTC 10654, NCTC 10656 and NCTC 10652. Three of the toxigenic isolates NCTC 10655, NCTC 11965 and NCTC 10657, showed higher binding to cells from non-secretors compared with binding to cells from secretors (Table 1). There was significantly higher binding of NCTC 10655 and NCTC 10657 to non-secretor cells at all ratios of bacteria: cells tested, while for strain NCTC 11965 there was significantly higher binding to cells of non-secretors only with the lowest ratio of bacteria.

Table 1. 95% confidence limits for binding of *Staphylococcus aureus* to non-secretor cells expressed as a percentage of binding to cells from matched secretors

| Strain no. | Toxin produced | Bacteria per cell |          |          |
|------------|----------------|-------------------|----------|----------|
|            |                | 80                | 160      | 320      |
| NCTC 8532  | —              | 66-145            | 69-146   | 31-182   |
| NCTC 10652 | A              | 63-198            | 63-227   | 50-301   |
| NCTC 10654 | B              | 67-153            | 75-185   | 75-206   |
| NCTC 10655 | C              | 138-228†          | 107-249* | 107-237* |
| NCTC 10656 | D              | 61-136            | 79-175   | 67-178   |
| NCTC 10657 | A, B           | 110-197*          | 101-161* | 117-165† |
| NCTC 11965 | A, TSST-1      | 102-201*          | 92-192   | 81-203   |
| 40654      | A              | 82-182            | 54-189   | 91-222   |
| 41206      | B              | 74-147            | 75-172   | 74-158   |

\*  $P < 0.05$ ; †  $P < 0.01$ .

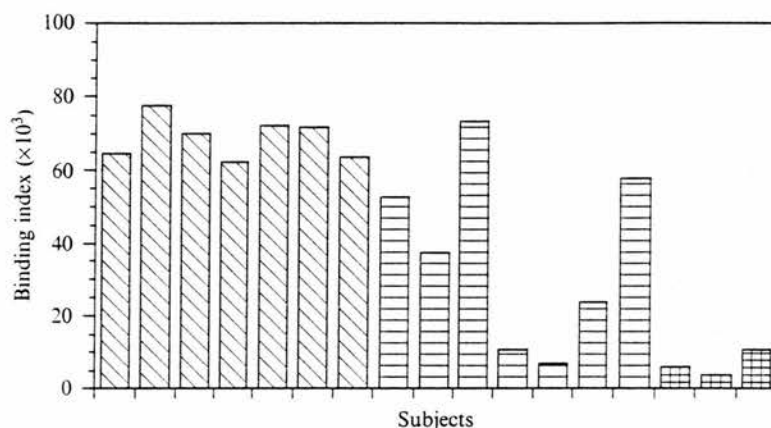


Fig. 1. Binding of monoclonal anti-Lewis<sup>a</sup> antibody to epithelial cells of non-secretors (▨), secretors (▤) and Lewis<sup>a-b-</sup> secretors (▩).

#### *Binding of anti-Lewis<sup>a</sup> or anti-precursor antibody to epithelial cells of secretors and non-secretors*

The epithelial cells from the three individuals whose erythrocytes were not agglutinated by either anti-Lewis<sup>a</sup> or anti-Lewis<sup>b</sup> (Lewis-negative) bound consistently low levels of anti-Lewis<sup>a</sup>. Cells from non-secretors bound high levels of the antibody. While the mean binding index for secretors (36707) was approximately half that for non-secretors (79148), there was considerable variation in the amount of antibody bound by individual secretors (Fig. 1). There was no consistent pattern of binding of the anti-precursor monoclonal to cells of secretors, non-secretors or Lewis-negative individuals.

#### *Inhibition of bacterial binding*

Binding of *S. aureus* NCTC 10655 to BEC obtained from 7 secretors of 7 non-secretors treated with anti-Lewis<sup>a</sup> antibody was significantly lower compared with binding of the bacteria to untreated cells from the same donors ( $t = -4.46$ ,

D.F. = 13,  $P < 0.001$ , 95% CI 55–81%). A similar pattern was observed for binding of NCTC 10655 to cells of 7 non-secretors and 10 secretors treated with monoclonal anti-precursor type 1 compared with untreated cells ( $t = 8.19$ , D.F. = 16,  $P < 0.001$ , 95% CI 58–72%).

Binding of NCTC 8532 to the cells from the same donors treated with anti-Lewis<sup>a</sup> antibody was significantly lower compared with binding of the bacteria to untreated cells ( $t = -4.97$ , D.F. = 11,  $P < 0.001$ , 95% CI 43–72%). Inhibition of NCTC 8532 with anti-precursor was not examined because of the limited amount of antibody.

*Binding of bacteria with reference to detection of Lewis<sup>a</sup> antigen or precursor antigen*

Binding of *S. aureus* NCTC 10655 was correlated with the amount of monoclonal anti-Lewis<sup>a</sup> antibody detected on the BEC of secretors and non-secretors (D.F. = 12,  $t = 5.03$ ,  $P < 0.001$ ). A similar pattern was found with NCTC 8532 ( $t = 4.24$ , D.F. = 12,  $P < 0.001$ ). There was no significant correlation between the amount of anti-precursor detected on epithelial cells and binding of NCTC 10655 or NCTC 8532.

*Binding of S. aureus to RSV infected cells*

Strains NCTC 8532 and NCTC 10655 were assessed in seven experiments to compare binding of bacteria to HEP-2 cells and RSV-infected HEP-2 cells. For both isolates the binding indices were greater for the virus infected cells: NCTC 8532 ( $P < 0.001$ , 95% CI 117–155%), NCTC 10655 ( $P < 0.05$ , 95% CI 109–155%).

## DISCUSSION

The results provided information for the three questions to be investigated in this study. Lewis<sup>a</sup> antigen was detected in secretions of 71% of SIDS infants examined. There was increased binding of some toxigenic strains of *S. aureus* to epithelial cells from non-secretor donors; and RSV-infected cells bound more staphylococci than uninfected cells.

The lack of relevant control populations is a major criticism of epidemiological studies of cot deaths. There is, however, evidence from several surveys that *S. aureus* is isolated from the nasopharynx of approximately 35–40% of SIDS infants [16, 18, 31] compared with 28% from healthy infants [18]. TSST-1 has been demonstrated in the renal tubular cells of some SIDS infants but not in a comparison group of infants who were not cot death victims [32]. The pyrogenic toxins of *S. aureus* and group A streptococci are powerful 'superantigens' that can induce release of cytokines that might trigger a cascade of events leading to shock or damage to the respiratory or cardiac systems [17].

Because density of colonization might be an important consideration in the hypothesis that these toxins play a role in some cot deaths, factors suggested to enhance colonization were assessed in the study reported here. Non-secretors of ABO blood group antigens are over-represented among carriers of group A streptococci [20]. We tested the hypothesis that epithelial cells from non-secretor donors might bind greater numbers of toxigenic staphylococci than cells from



secretors. Three of the eight toxigenic strains, including one producing TSST-1, did show significantly higher binding to non-secretor cells.

Attachment of strain NCTC 10655 which bound in greater numbers to non-secretor cells was significantly inhibited by pretreatment of the host cells with either monoclonal anti-Lewis<sup>a</sup> antibody or monoclonal anti-precursor type-1 antibody. Anti-precursor type 1 antibody was examined because it has been suggested that bacteria might bind to the precursor portion of the ABO or Lewis blood group antigens; and glycosylation of the precursor to H or Lewis antigens decreased the accessibility of the binding site [33]. There was a significant correlation between binding of NCTC 10655 and binding of anti-Lewis<sup>a</sup> antibody to the host cell, but there was no correlation between binding of this strain with the amount of anti-precursor antibody detected on the cells. As the anti-precursor monoclonal was prepared by immunization with Lewis<sup>a</sup> antigen, the inhibition of bacterial binding observed following pretreatment of cells with anti-precursor might be due to cross-reactivity with Lewis<sup>a</sup> although none was indicated by the manufacturer.

Although NCTC 8532 did not bind in greater numbers to non-secretor cells compared with secretor cells, its binding was inhibited by treatment of the epithelial cells with anti-Lewis<sup>a</sup> and there was a significant correlation between its binding to the donor cells and the amount of anti-Lewis<sup>a</sup> antibody bound by the cells. From Fig. 1, it is apparent that some secretors bind as much anti-Lewis<sup>a</sup> as most non-secretors. For the experiments comparing the binding of staphylococci to cells from secretors and non-secretors, the donors were matched only according to sex, age and ABO blood group; and the same pairs of secretor/non-secretor donors were not available for each experiment. At that time there was no information on the amount of anti-Lewis<sup>a</sup> bound by the cells of individual donors.

Early experimental work suggested that there are multiple receptors for staphylococci on human epithelial cells [34]. If Lewis<sup>a</sup> is one of the receptors for some strains of staphylococci or group A streptococci capable of producing pyrogenic toxins, the expression of this antigen among young infants might enhance their colonization by these bacteria. Viral infections have been shown to enhance carriage of staphylococci [24]; and disease due to TSST-1 has been reported to follow influenza or 'flu-like' illnesses [35]. Binding of both meningococci and type b *H. influenzae* to tissue culture cells derived from human epithelium (HEp-2) was substantially increased if the cells were infected with RSV [25]; and, in this study, both the toxigenic and non-toxigenic strain tested bound in greater numbers to the RSV infected cells. If there are similar interactions *in vivo*, infection with RSV common during the first year of life might also enhance colonization by these bacteria.

These studies suggest two factors that might enhance staphylococcal colonization of young infants; however, all infants who become colonized do not become SIDS victims. The following hypothesis is an attempt to correlate our laboratory findings with factors identified in epidemiological studies of cot deaths in New Zealand: mother's smoking, prone sleeping position and bottle feeding [4].

Mother's smoking which was associated with carriage of meningococci among children [36] might increase the risk of initial exposure to potentially pathogenic bacteria in two ways. First, epithelial cells from smokers have been shown to bind

greater numbers of staphylococci than cells from non-smokers [37]. Smoking also enhances susceptibility to respiratory viral infections; and epithelial cells from individuals with natural or experimental viral infections bound more staphylococci compared with those from individuals who were not infected with a virus [37].

In addition to the two factors examined in the present study, others that might enhance density of colonization of infants by staphylococci include passive exposure to cigarette smoke which decreases mucociliary clearance. Infants in the age range in which the peak of SIDS occurs have little or no mucosal or systemic immunity to staphylococci or to the pyrogenic toxins. The effect of breast feeding on carriage of staphylococci or susceptibility to these toxins is unknown; however, in studies on the possible role of toxigenic clostridia in cot deaths, *Clostridium difficile* was isolated from significantly fewer breast fed infants compared with formula fed infants, and *Cl. difficile* toxin was detected only in the faeces of formula-fed infants [38].

The pyrogenic toxins are produced between 37–40 °C; and they are produced in greater quantities at the higher temperatures [17]. Three factors associated with SIDS might increase the infant's temperature and thereby enhance toxin production: respiratory infection; overwrapping with clothing or bedding; and the prone sleeping position. In the prone sleeping position, infants lose less heat than in the supine position. [39–41]. The synergistic effect between increased temperature and increased toxin production might account for the high temperatures recorded for some of these infants at autopsy. Among 24 infants who died suddenly and whose rectal temperatures were measured immediately before refrigeration, 10 had temperatures > 38 °C and 5 were > 40 °C [42]. In addition to the heat-shock hypothesis of cot death, the increased release of IL-1 induced by the toxins might contribute to prolonged sleep apnoea as suggested by Guntheroth [43].

Studies are underway to assess levels of *Lewis*<sup>a</sup> in saliva of infants, exposure to cigarette smoke and presence of RSV on carriage of toxigenic staphylococci and streptococci. Results of these studies should provide evidence to refute or confirm the scheme proposed.

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### Isolation of an adhesin from *Staphylococcus aureus* that binds Lewis <sup>a</sup> blood group antigen and its relevance to sudden infant death syndrome <sup>1</sup>

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# Isolation of an adhesin from *Staphylococcus aureus* that binds Lewis<sup>a</sup> blood group antigen and its relevance to sudden infant death syndrome<sup>1</sup>

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**Abstract:** A 67 kDa protein was isolated from cell membrane preparations of *Staphylococcus aureus* (NCTC 10655) by affinity adsorption with synthetic Lewis<sup>a</sup> antigen conjugated to Synsorb beads. Pre-treatment of buccal epithelial cells expressing Lewis<sup>a</sup> with the purified protein reduced binding of the staphylococcal strain to a greater extent than the material not bound to the Synsorb beads. The significance of this work is discussed with reference to expression of Lewis<sup>a</sup> antigen in infants and the proposed role of toxigenic strains of staphylococci in some cases of sudden infant death syndrome.

**Key words:** *Staphylococcus aureus*; Adhesin, Lewis<sup>a</sup>; Blood group antigen; Sudden infant death syndrome

## Introduction

The role of microorganisms in the aetiology of sudden infant death syndrome (SIDS) has been investigated over many years, but the evidence that a single microorganism is responsible for these infant deaths remains inconclusive. Several of the factors identified in epidemiological studies of SIDS are associated with increased susceptibility of infants to infectious diseases. SIDS

occurs most commonly in the vulnerable period when maternal antibodies in the infant have decreased and the immune system is immature. SIDS also occurs more frequently during the winter months when respiratory viral infections are most prevalent. These infections are likely to be exacerbated by exposure to cigarette smoke, and mother's smoking has been identified as a risk factor in SIDS. Breast-fed infants are not only less vulnerable to gastro-intestinal and respiratory infections but also less susceptible to SIDS [1].

By definition, invasive bacterial diseases are not the cause of SIDS, but some studies have suggested a role for toxin-producing bacteria such

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<sup>1</sup> Patent for isolation of adhesins that bind Lewis<sup>a</sup> applied for, Patent Office No. 9312097.0.

as *Clostridium botulinum* [2,3], *Escherichia coli* [4-7] and *Staphylococcus aureus* [4,8-10]. Some strains of *S. aureus* and group A *Streptococcus pyogenes* [11] produce pyrogenic toxins which act as superantigens that have significant physiological effects on the host, e.g. toxic shock syndrome.

Epidemiological studies have found that individuals who are non-secretors of the ABO blood group antigens are over-represented among patients with some bacterial diseases and among carriers of some potentially pathogenic bacteria. The Lewis<sup>a</sup> antigen, usually present in greater quantities on epithelial cells of non-secretors, might be a receptor for some adhesins on strains of these bacterial pathogens. If so, individuals who express significant amounts of Lewis<sup>a</sup> might be more readily or more densely colonized by these organisms [12,13].

Previous studies have shown that binding of both toxigenic and non-toxigenic strains of *S. aureus* to epithelial cells was correlated with the amount of Lewis<sup>a</sup> antigen detected on the cells, and monoclonal antibody to this antigen can block attachment of these bacteria [14]. In this study an attempt was made to isolate the surface component of *S. aureus* that binds Lewis<sup>a</sup> and to use it to reduce attachment of the bacteria to epithelial cells.

## Materials and Methods

### Bacterial strains

Lyophilized *S. aureus* strains NCTC 10655, NCTC 10652 and NCTC 8532 were obtained from the Central Public Health Laboratory, Colindale. Bacteria were cultured on nutrient agar for 24 h at 37°C. Colonies were emulsified in Microbank beads and kept at -20°C for further use. A fresh bead was used to inoculate plates for each experiment.

### Cell wall isolation

A modification of the method described by Cheung et al. [15] and Sharp and Poxton [16] was used. Colonies were suspended in washing buffer (0.05 M Tris-HCl, 0.025 M MgCl<sub>2</sub> and 0.15 M NaCl, pH 7.6) and centrifuged for 20 min at

2000 × *g* at 4°C. The bacterial pellet (2 g) was suspended in freshly prepared washing buffer containing DNase 1 (50 µg ml<sup>-1</sup>) (BDH, 39012) and RNase (50 µg ml<sup>-1</sup>), (Calbiochem, lot 902237). The pre-cooled (4°C) cell suspension was passed twice through a French Pressure Cell (Aminco American Instrument Co., Silver Springs, MD) at 10<sup>7</sup> pa = 6000-7000 lb/in<sup>2</sup> and centrifuged at 2000 × *g* for 20 min at 4°C to remove any intact bacteria. The supernate was centrifuged at 20 000 × *g* for 30 min to recover the cell wall material. The resulting pellet was washed 3 times in ice-cold distilled water by centrifugation at 30 000 × *g* for 15 min at 4°C.

### Extraction of cell wall protein

The pellet from the crude cell wall preparation was suspended at room temperature in 5 ml of 2% (v/v) Triton X-100 (Sigma T-6878) in water for 30 min and centrifuged at 20 000 × *g* for 20 min. After two such extractions, the pellet was dissolved in 5 ml of a freshly prepared solution containing 50 µg ml<sup>-1</sup> lysostaphin (Sigma L-7386) in 0.05 M Tris-HCl with 0.145 M NaCl (pH 7.6) and incubated at 37°C for 2 h with continuous shaking. After centrifugation at 45 000 × *g* for 20 min at 4°C, the supernate was freeze-dried, reconstituted in distilled water and stored at -20°C.

### Polyacrylamide gel electrophoresis (PAGE)

The buffer system of Laemmli [17] with 10% w/v acrylamide in a Biometra-Minigel (Biometra, Wagen-Stieg, Göttingen, FRG) was used. Protein content was determined by the method of Bradford [18] with bovine serum albumin as standard. Samples (20 µl) containing 8 µg protein were applied to the gel, run for 1-2 h at 60 V for the stacking gel and 150 V to complete the run and stained with Coomassie brilliant blue. Molecular mass markers for molecular masses 30 000 to 200 000 (Sigma, mw-SDS-200) were run with each gel.

### Purification of adhesins by affinity adsorption

Synthetic Lewis<sup>a</sup> covalently bound to Synsorb affinity adsorbent (100 mg) (ChemBiomed Ltd., Edmonton, Alberta, Canada; lot ASi-137) was swollen in a Falcon tube in 2 ml of phosphate



buffered saline (PBS) (pH 7.2) for 2 h at room temperature with continuous rotation. The beads were centrifuged at  $50 \times g$  for 5 min and the supernate removed. The protein extract (0.17 mg in 1 ml) from strain NCTC 10655 was added to the beads, rotated overnight at  $4^\circ\text{C}$  and centrifuged at  $50 \times g$  for 5 min. The supernate containing unbound material was collected. The beads were washed twice with 2 ml of PBS and the washings added to the unbound material. The bound material was eluted from the beads with 1 ml of 2% (v/v) ammonia (25% solution, BDH) in saline for 20 min with continuous rotation at room temperature. The supernate was recovered by centrifugation at  $50 \times g$  for 5 min. The beads were finally washed with 2 ml of PBS and the eluate added to the previous supernate. The original sample and all extracts were freeze-dried, reconstituted in distilled water and dialysed overnight against Tris-HCl (0.01 M, pH 7.4) at  $4^\circ\text{C}$ .

#### Inhibition assay

The affinity purified extract (protein) and the unbound material of NCTC 10655 prepared as above were made up to a protein concentration of  $15 \mu\text{g ml}^{-1}$ . A volume ( $100 \mu\text{l}$ ) of each was added to  $100 \mu\text{l}$  of suspensions of buccal epithelial cells (BEC) ( $2.5 \times 10^5 \text{ ml}^{-1}$ ) taken from 2 healthy individuals expressing Lewis <sup>a</sup>. Cells incubated with  $100 \mu\text{l}$  of PBS were included as untreated controls. The mixture was incubated for 1 h at room temperature. The cells were washed twice with PBS by centrifugation at  $300 \times g$  for 10 min. *S. aureus* NCTC 10655 cells ( $100 \mu\text{l}$ ) labelled with fluorescein isothiocyanate (FITC) by the method described previously [14] were added to the BEC at a ratio of 640 bacteria per cell. After 30 min at  $37^\circ\text{C}$  with continuous shaking, the cells were washed twice with PBS, fixed with 1% v/v paraformaldehyde and analysed by flow cytometry in an EPICS C (Coulter Ltd., UK) as previously described [14]. The binding index (BI) for each sample was calculated by multiplying the percentage of fluorescent cells by the mean fluorescence channel of the positive population. The percent inhibition was calculated by the formula  $100 - [(BI \text{ test} / BI \text{ control}) \times 100]$ .

## Results

#### Cell wall preparations

The protein profiles by PAGE of the material solubilised by lysostaphin from strains NCTC 10655, NCTC 10652 and NCTC 8532 are shown in Fig. 1. The band at 28 kDa is the lysostaphin. All three strains showed numerous common bands.

Figure 2 shows the peptide bands of strain NCTC 10655 before and after adsorption with Synsorb Lewis <sup>a</sup>. The bands for the unbound material (track 2) and the original cell wall material

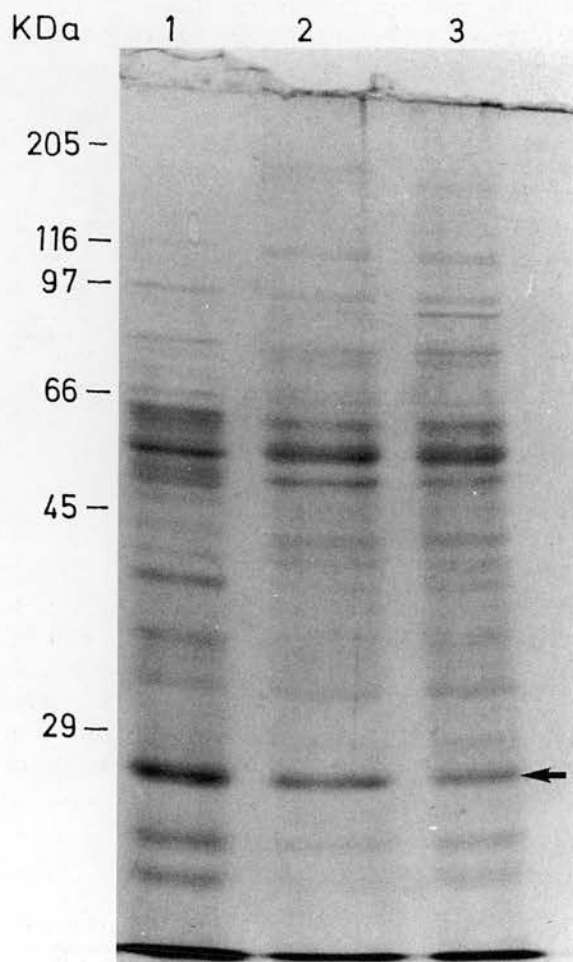


Fig. 1. Coomassie brilliant blue-stained SDS-PAGE (10%) of proteins ( $8 \mu\text{g}$ ) extracted from *S. aureus* NCTC 10655 (lane 1), NCTC 10652 (lane 2), NCTC 8532 (lane 3).

(track 1) show similar patterns of multiple bands while the eluate (track 3) has a major band of approximately 67 kDa. The protein concentration of the original cell wall extract was  $170 \mu\text{g ml}^{-1}$  and that of the unbound material and Synsorb eluate were  $150 \mu\text{g ml}^{-1}$  and  $15 \mu\text{g ml}^{-1}$  respectively.

#### Inhibition studies

The ability of the protein preparations to inhibit binding of *S. aureus* NCTC 10655 to buccal epithelial cells was assayed by flow cytometry. In each of 3 experiments, the percentage inhibition

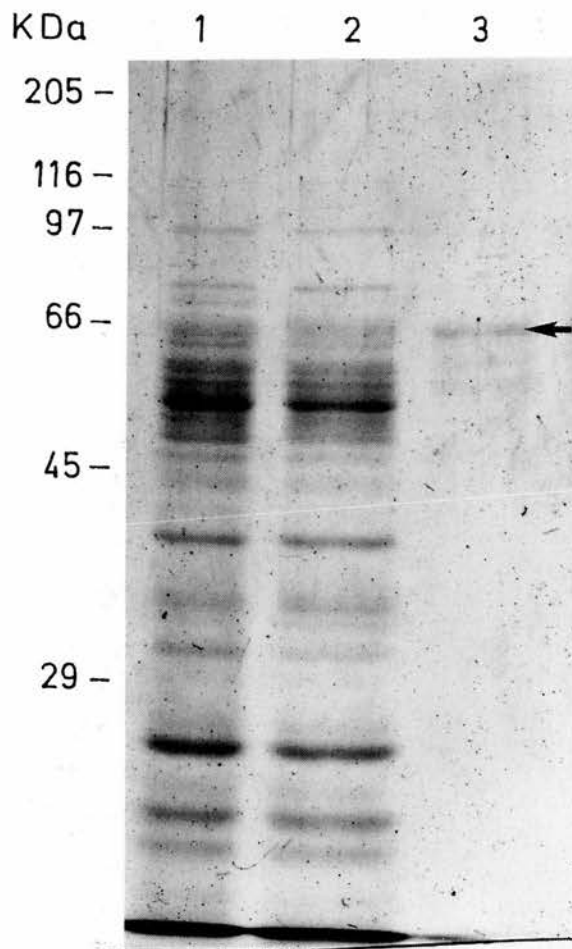


Fig. 2. Coomassie brilliant blue-stained SDS-PAGE (10%) of cell wall extracts of *S. aureus* NCTC 10655 (lane 1), unbound material (lane 2), and Synsorb-Lewis<sup>a</sup> eluate (lane 3).

Table 1

Inhibition of binding of *S. aureus* NCTC 10655 by pretreatment of buccal epithelial cells (BEC) with protein preparations eluted from Synsorb-Lewis<sup>a</sup> and the unbound material

| Experiment | % Inhibition                                  |  |  |
|------------|---|--|--|
|            | Whole extract<br>( $15 \mu\text{g/ml}^{-1}$ ) | Unbound<br>protein<br>( $15 \mu\text{g/ml}^{-1}$ ) | Synsorb-Lewis <sup>a</sup><br>eluate<br>( $15 \mu\text{g/ml}^{-1}$ ) |
| 1          | 44.8  | 25.5   | 54.8   |
| 2          | ND *  | 6.5  | 24   |
| 3          | ND  | 13.3   | 25.7   |

\* ND = not done.

was greater for the protein eluted from Synsorb Lewis<sup>a</sup> (Table 1). The eluate from a control preparation obtained by passing lysostaphin solution through the Synsorb beads showed no bands and no inhibition of bacterial binding to the buccal epithelial cells.

#### Discussion

The Synsorb Lewis<sup>a</sup> affinity purification isolated a major polypeptide of 67 kDa from cell wall preparations of *S. aureus*. This supports our previous work which has shown that binding of *S. aureus* strains to buccal epithelial cells was significantly correlated with the amount of Lewis<sup>a</sup> on the cells and binding of both NCTC 10655 and NCTC 8532 to these cells could be significantly reduced by pre-incubation of the cells with monoclonal anti-Lewis<sup>a</sup> [14]. The results also complement the studies in which these strains were agglutinated by anti-idiotypic antibodies produced by immunization of mice with monoclonal anti-Lewis<sup>a</sup> [19].

The cell wall preparations were capable of inhibiting attachment of strain NCTC 10655 to buccal epithelial cells; the inhibitory activity of the material eluted from Synsorb Lewis<sup>a</sup> was greater than that of the unbound material. Complete inhibition of binding was not predicted as Gram-positive bacteria express multiple adhesins [20,21]. Teichoic acids, protein A and fibronectin-binding proteins have been implicated in adherence [20,22,23]. The molecular mass of the

Table 2  
ABO and Lewis blood group antigens present on cells and in body fluids of secretors and non-secretors

| Antigen            | Secretor |             | Non-secretor |             |
|--------------------|----------|-------------|--------------|-------------|
|                    | Cells    | Body fluids | Cells        | Body fluids |
| H                  | +        | +           | +            | —           |
| A/B                | +        | +           | +            | —           |
| Lewis <sup>a</sup> | +/-      | +/-         | +            | +           |
| Lewis <sup>b</sup> | +        | +           | —            | —           |

Lewis <sup>a</sup> adhesin (67 kDa) differs from those reported for fibrinogen or fibronectin-binding proteins: 18 kDa [24]; 19 kDa [25]; 62 kDa [26]; and 200 kDa [27].

Lewis antigens are present on epithelial cells of approximately 95% of most human populations. Expression of the Lewis antigens, Lewis <sup>a</sup> and Lewis <sup>b</sup>, is modified by the secretor gene (*Se*); both the *Se* and Lewis (*Le*) genes are in the same linkage group on chromosome 19. Individuals who are secretors, homozygous (*Se, Se*) or heterozygous (*Se, se*) for the secretor gene, will have the type 1 form of their ABO blood group antigens in their body fluids. Those of the 'recessive' non-secretor genotype (*se, se*) do not have type 1 ABO antigens in their body fluids (Table 2).

Production of Lewis antigen is dependent on fucosyl transferases coded for by the *Se* gene and the *Le* genes. Both enzymes add fucose to type 1 precursor chains from which most of the ABO and Lewis antigens in secretions derived. If the *Se* transferase adds fucose to the terminal sugar of the precursor chain, the *Le* enzyme can add fucose to the subterminal sugar to produce Lewis <sup>b</sup>. If the *Le* enzyme adds fucose to the subterminal sugar first to produce Lewis <sup>a</sup>, the secretor enzyme cannot use this structure as a substrate, and Lewis <sup>a</sup> is the final product [28]. In infants, the fucosyl transferase coded for by the *Se* gene is less efficient than that coded for by the *Le* gene; consequently, infants express easily detectable amounts of Lewis <sup>a</sup> even though the amount might be greatly reduced as they become older.

If Lewis <sup>a</sup> antigen is predominately expressed on the cells of infants' during the first months of

life [29], this might contribute to their colonization by bacteria expressing adhesins that bind to Lewis <sup>a</sup>. The incidence of SIDS closely parallels the expression of the Lewis <sup>a</sup> antigen in infants [30,31] and isolation of staphylococci from infants [32]. Colonization by toxigenic staphylococcal strains in such circumstances would not necessarily lead to SIDS. Other environmental factors, e.g. virus infection, or overheating, are probably needed to trigger the toxin production and the series of events leading to cot deaths [31].

Studies with the anti-idiotypic reagent suggest adhesins that bind Lewis <sup>a</sup> are present on a significant proportion of other species that affect infants (*Neisseria meningitidis* and type b *Haemophilus influenzae*) [19]. The method described here is being adapted to isolate the Lewis <sup>a</sup> adhesin from Gram-negative bacteria.

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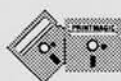
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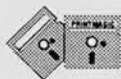
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